Studies on the Role of Blood Group Antigens in Susceptibility to Meningococcal Infection

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Ph.D.

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1990

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Declaration

I declare that this thesis has been composed by myself and that the research reported therein has been conducted by myself or under my direct supervision.

Edinburgh, 23 July 1990.

Ayelet Rahat

I dedicate my work to the memory of my parents

Acknowledgments

First and foremost I wish to express my gratitude to my dedicated supervisors: Dr. John Stewart, Dr. Caroline Blackwell and Prof. Donald Weir. It has been a great pleasure to be instructed by them, and their enthusiasm and encouragement was of much help throughout these years. It is thanks to the long hours of patient discussions they have spent with me that the completion of this thesis was made possible. I would also like to mention their understanding and friendly attitude that helped much in bringing up my first child, alongside with writing up my first thesis... Thank you.

I also wish to thank my colleagues in the Infection & Immunity and the Immunology laboratories for providing pleasant company. In particular, many thanks to Mike Kerr, Doris MacKenzie and Valerie James for assisting me both with carrying out experiments, and with the nagging process of typing and proof-reading this thesis. Thanks are also due to Mrs. D. Harkiss and Mr. W. Neill for their helpful attitude in reading experiments on the EPICS. More thanks to colleagues at the Bacteriology Department for kindly donating samples for my experiments.

I am grateful to Dr. R.H. Fraser and Dr. G. Inglis of the Glasgow and West of Scotland Blood Transfusion Services for helpful discussions and generous supply of antibodies. Their contribution has been an essential ingredient for carrying out this research project.

I am most grateful to the funding bodies that supported this research project: the Meningitis Trust and TENOVUS, Scotland. The latter have put enormous efforts raising the funds, for which I would like to express my appreciation.

A different kind of feelings and thanks I wish to express to my supporting family: Tal, Ana and Menachem, and Talma and Gavri. Though so distant, their love, as well as actual assistance, was more than essential.

Last but not least, I would like to thank my beloved husband, Udi, and my son, Shye. Shye, for being a constant reason for joy; and Udi, for his patience and support throughout these years. His help was both in stimulating discussions and with the tiring process of producing this thesis.

Abstract

Non-secretors of ABO blood group antigens are over-represented among patients with disease due to *Neisseria meningitidis* and among carriers of this bacterium. On the basis of the known differences between secretors and non-secretors, two hypotheses have been suggested to explain this epidemiological observation. The first suggests that the H blood group determinant is a binding site for bacteria. It is proposed that the presence of this structure in body fluids of secretors can block the attachment of the bacteria to cells, reducing colonization. The second hypothesis suggests that Lewis^a (Le^a) blood group determinants, found mainly on cells of non-secretors, acts as a receptor for the bacteria. The aim of this study was to investigate whether Le^a or H determinants might be receptors for *N. meningitidis.*

As a preliminary step, the differential distribution of H determinants in saliva and on cells of secretors and non-secretors was studied. An enzyme linked immunosorbant assay (ELISA) was developed for the measurement of H determinants in secretions. With this method, the level of H determinants in secretions of secretors was found to be higher than in secretions of non-secretors. The level of H determinants on the surface of epithelial cells was determined by flow cytometric analysis. Between 3.5-6 times more H epitopes were detected on epithelial cells from secretors compared with cells from non-secretors. With the same technique, uptake of antigens containing H Type 1 determinants from saliva onto epithelial cells was demonstrated.

The level of attachment of *N. meningitidis* to epithelial cells obtained from secretors and non-secretors was studied by flow cytometry. At a low ratio of bacteria to epithelial cells, more bacteria bound to cells from non-secretors com-

pared with cells from secretors. The finding that the level of H determinants did not positively correlate with the amount of bacteria attached suggests that H is not a receptor for the bacteria.

A possible role for the Le^a determinant as a binding site for *N. meningitidis* was then investigated. The binding of meningococci to cells of non-secretors was inhibited by preincubation of the bacteria with affinity purified molecules containing Le^a determinants. Using an ELISA developed for the detection of Le^a determinants, a number of *N. meningitidis* isolates were found to adsorb affinity purified molecules containing Le^a epitopes. These results support the hypothesis that Le^a is a receptor for the bacteria.

Finally, an attempt was made to identify the structure on the bacteria and the carbohydrate sequence of the blood group determinant that interacts in the attachment process. The binding of synthetic glycoproteins containing blood group determinants to preparations of outer membrane proteins from *N. meningitidis* strain (C:2b:P1.2) was demonstrated. Human serum albumin conjugated with Type 1 blood group structures (precursor chain, H, $Lewis^a$ and $Lewis^b$) were found to bind to a preparation of the outer membrane proteins of *N. meningitidis*. Precursor Type 1 chain is the carbohydrate structure common to all glycoconjugates used, suggesting that this structure is part of the bacterial binding site. Greater accessability of the precursor Type 1 chain receptor on antigens of non-secretors compared with secretors, could contribute to the increased susceptibility of non-secretors to meningococcal disease.

Contents

Chapter 1

Introduction

Studies of the associations between blood group phenotypes and susceptibility to disease began soon after the discovery of the ABO blood groups at the beginning of this century. These studies have been reviewed in "Blood Groups and Diseases" (Mourant *et al.*, 1978) and more recently by Bird (1983) and Blackwell (1989b).

A phenomenon related to the blood group systems is the secretion of A, B and H blood group structures into body fluids. This genetically determined feature also influences the type of Lewis blood group determinant present on cells and in secretions. The ability to secrete the ABH blood group antigens was found to be a susceptibility factor for several diseases reviewed by Blackwell (1989b). Over the last few years a number of epidemiological surveys have demonstrated that non-secretors of the ABH blood group antigens are more susceptible to infections and to some autoimmune conditions. This study was initiated to examine hypotheses suggested to explain the association of non-secretion of blood group antigens with meningococcal disease and carriage of these bacteria.

In this chapter four general areas are reviewed to provide necessary background for the approach used in this study: 1) biochemistry and genetic control of blood group antigens related to secretor status; 2) blood groups and disease susceptibility; 3) the epidemiology and pathogenesis of meningococcal disease; 4) hypotheses proposed to explain the increased susceptibility of non-secretors to infectious diseases. The aims of this study, to examine two hypothesis relating to colonization of epithelial surfaces by *N. meningitidis*, are summarised at the end of this chapter.

1.1 The ABO and Lewis Blood Group Systems and Secretor Status

1.1.1 Introduction

Agglutination of red blood cells from one species by serum from another was discovered by Londois in 1875 (reviewed by Race and Sanger, 1975). Twenty five years later, Landsteiner noted that human red blood cells can be agglutinated by serum from another individual. This observation led him to the discovery of the ABO blood group system (see Mourant *et al.*, 1978). Within a few years studies showed that humans could be divided into four groups (A, B, O and AB) according to the reaction of their red blood cells with normal human sera. This information was later adopted for blood transfusion purposes which started at the end of World War I. In 1910, Dunger and Hirszfeld demonstrated that the blood group antigens were inherited as Mendelian characters (reviewed by Mourant *et al.*, 1978).

In 1946, Mourant reported the discovery of anti-Le^a. The antibody described reacted with red cells and was recognized as defining a new antigen. In 1948, Andersen described the first example of anti-Le^b (see Race and Sanger, 1975). It is now known that these antigens are not the products of allelic genes, but a result of the interaction between two gene products.

In this part of the introduction the biochemistry and genetics of the ABH and the Lewis blood group systems will be reviewed as they are essential for examination of the association between secretion of blood group antigens and disease.

1.1.2 The ABO System

Biochemistry

The ABH blood group determinants are carbohydrate chains carried on macromolecules, mainly glycoproteins in secretions and glycolipids and glycosphingolipids on cell surfaces. The carbohydrate moiety of all blood group antigens is composed of a basic precursor chain to which different monosaccharides are added by different glycosyltransferases. These enzymes are suggested to be the primary protein product of the blood group genes. The monosaccharides added by the glycosyltransferases defines the serological properties of the different blood group determinants.

Four types of precursor chains that carry the ABH antigens have been described (Rege *et al.*, 1963; Donald, 1981; Bremer *et al.*, 1984). Of these, antigens derived from Type 1 and Type 2 precursor substances appear to carry most of the ABH antigens on cells and in secretions. Type 1 chains have a D-galactose residue joined by a β 1–3 linkage to N-acetyl-D-galactosamine , whereas the Type 2 chain has a β 1–4 linkage between these two sugars (Figure 1.1). The structure of H (Rege *et al.*, 1964; Lloyd *et al.*, 1966), A and B (Painter *et al.*, 1965; Lloyd *et al.*, 1966) determinants based on Type 1 and Type 2 chains are shown in Figure 1.2. H Type 1 and 2 are composed of the respective precursor chains with an additional L-fucose joined to the C2 position of the terminal D-galactose residue. The A and B determinants are based on the H structure with an additional monosaccharide: N-acetyl-D-galactosamine in A determinants and D-galactose in B determinants. The biochemical pathways by which the ABH antigens are formed is shown in Figure 1.3. Each one of the blood group genes codes for a glycosyltransferase required for the synthesis of the corresponding

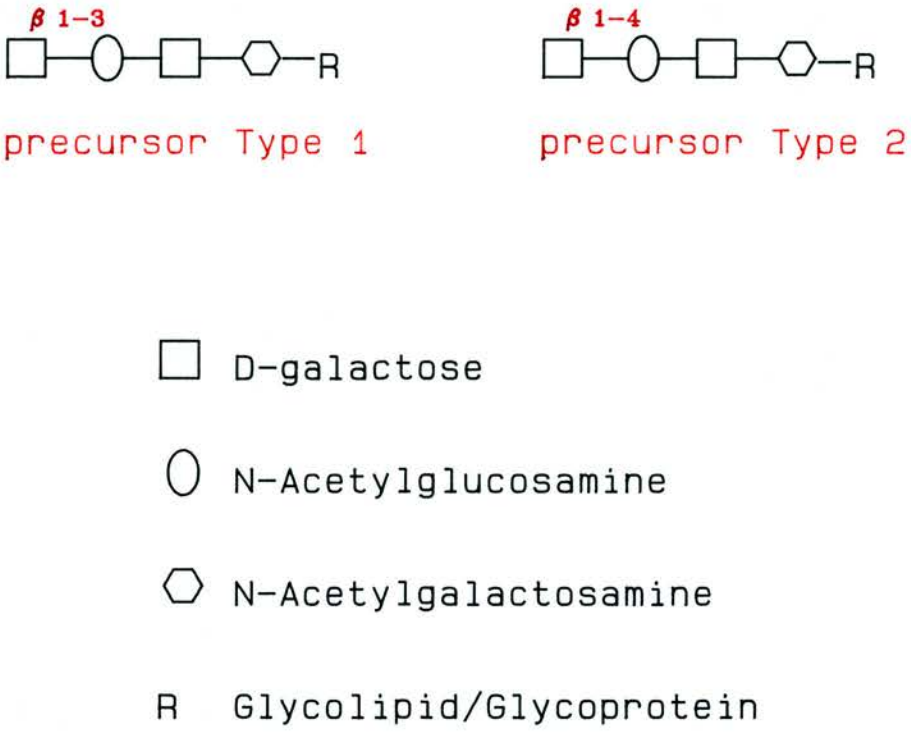

Figure 1.1: Diagram of the two types of carbohydrate chain endings which form the basis of A, B and H determinants on cells and in secretions.

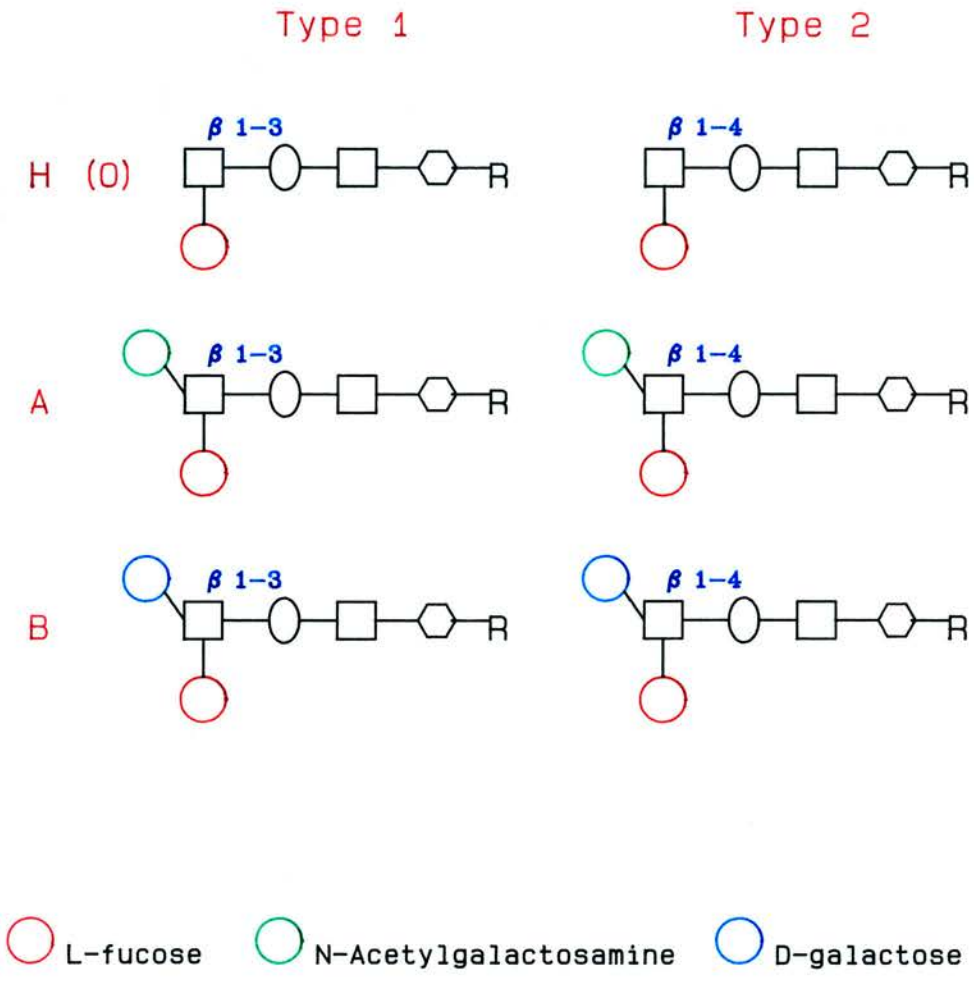

Figure 1.2: Structure of H, A and B determinants based on Type 1 and Type 2 carbohydrate chains. The symbols for the constituents of precursor chains are as in Figure 1.1.

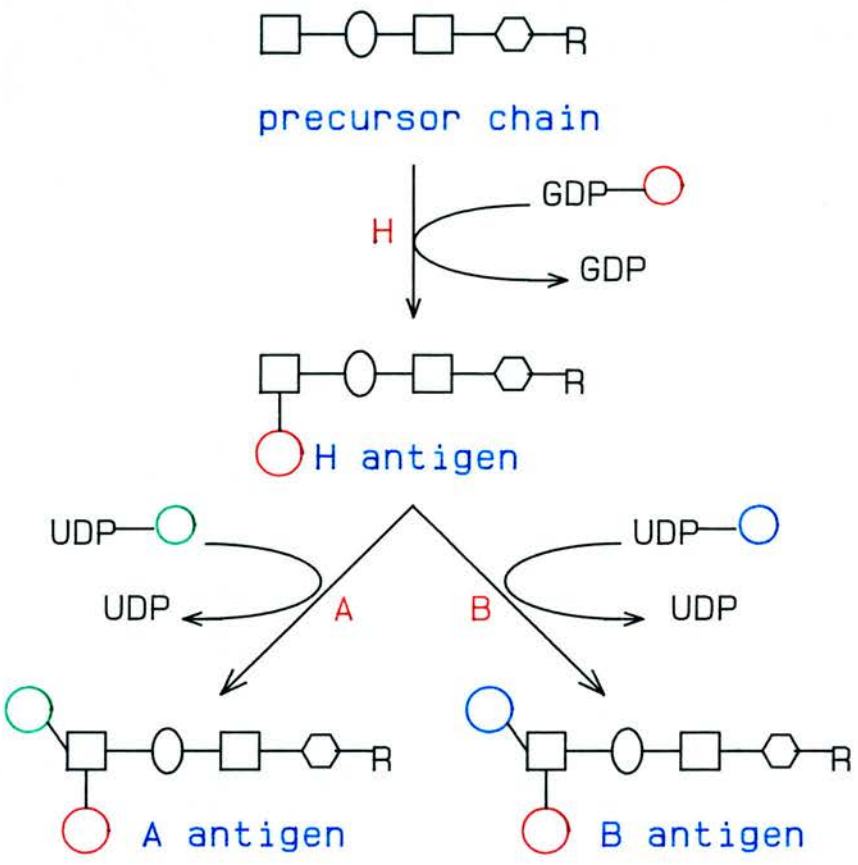

Figure 1.3: Biochemical pathways for the formation of H, A and B structures. Symbols as in Figures 1.1 and 1.2. The H transferase (H) specifically transfers fucose from guanosine diphosphate (GDP) L-fucose to precursor chain. The A transferase (A) specifically transfers N-acetylgalactosamine from uridine diphosphate (UDP) N-acetylgalactosamine to the H structure. The B transferase (B) specifically transfers galactose from uridine diphosphate (UDP) D-galactose to the H structure.

blood group determinant. These enzymes catalyze the transfer of a specific nucleotide sugar to an acceptor structure on a glycolipid or glycoprotein molecule (Watkins, 1967).

In secretions and in endodermally derived tissues such as lining epithelia, the blood group determinants are carried mainly on Type 1 chains (Oriol *et al.*, 1986). A prime necessity for the formation of the terminal disaccharide of a Type 1 structure is the expression of an N-acetylglucosamine β-3-galactosyltransferase. This enzyme has not been isolated or purified, and very little is known about its distribution in human tissues.

The endogenously produced ABH antigens expressed on erythrocytes are based on Type 2 chains. Small quantities of ABH determinants carried on Type 1 chain can be found on red blood cells. These antigens are adsorbed onto the surface of the cells from the plasma (Tilley *et al.*, 1975).

Blood group determinants are known to be carried on a variety of lipid and protein carriers. They have been reported to appear as part of the epidermal growth factor receptor (Parker *et al.*, 1984) and on various hydrolytic enzymes (Green *et al.*, 1988). The carbohydrate chain carried on these macromolecules can vary in length and complexity from a short simple chain to complex highly branched structures. Variation in the carrier (lipid/protein) molecules and in the carbohydrate residues, give rise to a wide range of molecules expressing blood group determinants. The molecular weight of antigens carrying blood group determinants in secretions has been estimated to range from $2x10^5$ to several million daltons (Watkins, 1980).

The term blood group antigen is used to refer to a group of molecules, all carrying the same carbohydrate structure at the end of the oligosaccharide chain

and able to induce an allogeneic immune response to this determinant. A great variety of molecules can carry the same carbohydrate structure; therefore, each one of these molecules can be considered as a separate antigen sharing one antigenic determinant. To be accurate, the term blood group determinant should be used to describe a carbohydrate structure responsible for the production of isohaemagglutinins.

Genetic control

The ABO locus present on chromosome 9 (Westerveld *et al.*, 1976) was first thought to contain three major alleles: *A*, *B* and *O* (reviewed by Watkins, 1980). It is now known that there are two *A* alleles encoding for A_1 and A_2, the two subgroups of blood group A (reviewed by Watkins, 1980). One of the four alleles is transferred to the offspring from each parent. The *O* allele does not give rise to a functional product therefore the three functional alleles are A_1, A_2 and *B*. Each gene codes for a glycosyltransferase that adds the appropriate sugar for the formation of the specific blood group determinant. The term H was introduced by Morgan and Watkins (1948) to describe the antigen found in all ABO phenotypes. This substance is synthesised by a glycosyltransferase coded by the *H* gene. The *H* gene is situated at a site independent of the ABO locus and produces the substrate for the products of the *A* and *B* genes (Watkins and Morgan, 1955). The *h* gene is a rare silent allele of *H*. Individuals homozygous for *h* (*hh*) are the para-Bombay phenotype in whom blood group antigens are not expressed in tissues of ectodermal origin. Since the H determinant is the acceptor for the A and B enzymes, in para-Bombay individuals these determinants will not be formed on Type 2 precursor chains, although the A and B transferases may be present (see Figure 1.4).

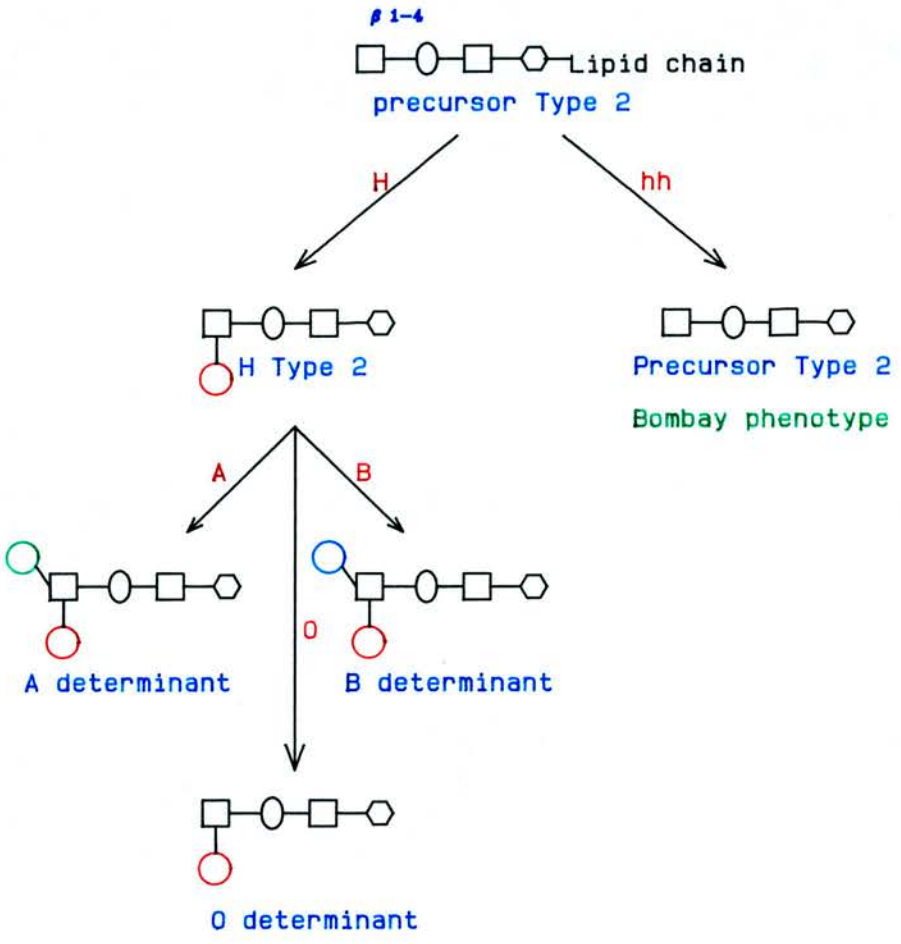

Figure 1.4: Diagram showing the production of A, B and H determinants on Type 2 chains. Symbols as in Figures 1.1 and 1.2. The arrows represent the reactions possible in individuals possessing the genes indicated by the letters.

The ability to produce blood group determinants in secretions is determined by allelic genes, *Se* and *se* , on chromosome 19 and is inherited as a Mendelian dominant character (see Watkins, 1980). An individual homozygous for the *Se* gene (*SeSe*) or heterozygous (*Sese*) is a "secretor"; whereas, an individual homozygous for the silent allele (*sese*) is a "non-secretor". Among Europeans, about 80% are secretors and 20% are non-secretors. These percentages can vary among different ethnic groups (Race and Sanger, 1975).

Two hypotheses have been proposed to explain the role of the *Se* gene in determining secretor status. On the basis of biochemical studies it has been suggested that the *Se* gene is a regulatory gene, controlling the expression of the *H* gene in epithelial tissues (Watkins, 1959). This hypothesis received support from the observation that the α-2-fucosyltransferase (the enzyme coded by the *H* gene) is strongly expressed in glandular and mucosal tissues from secretors and only minimally in those of non-secretors (Ginsburg, 1972).

A second hypothesis more recently proposed by Oriol *et al.*, (1981) suggests that the *Se* gene is a structural gene. This "two structural gene" model states that a second α-2-fucosyltransferase, coded by the *Se* gene, is responsible for the formation of the H structure in tissues of endodermal origin. The enzymes coded by the *H* and *Se* genes transfer L-fucose to a terminal β-galactose to form the H determinant; but they differ in the precursor chain used as an acceptor. It is suggested that the enzyme coded by the *H* gene preferentially fucosylates Type 2 precursor chain; whereas the *Se* gene product has a higher affinity for the Type 1 precursor chain than for Type 2. This proposal is supported by several studies. In the first, fucosyltransferase from human milk, which is associated with possession of the *Se* gene, showed 20 fold higher affinity for Type 1 compared with Type 2 chains (Kumazaki and Yoshida, 1984). In another study, a partially

purified fucosyltransferase from submaxillary glands of secretors showed distinct preference for the Type 1 precursor chain. This preference was not found for the weak enzymatic activity found in submaxillary glands from non-secretors (Betteridge and Watkins, 1985). This "two structural gene" model explains the existence of the para-Bombay phenotype where the H determinant is found in secretions but not on erythrocytes. In para-Bombay individuals, the *Se* gene is expressed and H is present in body fluids, but both alleles in the *H* locus are silent (*hh*), therefore H is absent from tissues of ectodermal origin (including red blood cells). In Bombay individuals both genes are inactive and the H determinants are not found on cells or in secretions (Oriol *et al.*, 1986).

The *Se* and *H* genes are closely linked on the short arm of chromosome 19 (Oriol *et al.*, 1981); and it has been suggested that one of the genes might have been derived by gene duplication in the course of evolution (Le Pendu *et al.*, 1985).

1.1.3 The Lewis system

Biochemistry

Lewis antigens on red blood cell surfaces differ from the ABH antigens in that they are not synthesized endogenously by the erythrocytes but passively acquired from the plasma (Sneath and Sneath, 1955; Marcus and Cass, 1969). The site of production of the Lewis antigens which circulate in the plasma is unknown. It is also not known if there are specific receptors on the cells for glycolipids carrying the Lewis determinants or if they are non-specifically adsorbed into the lipid bilayer of the cells membrane. The *Lewis* gene product is

an α-4/3-L-fucosyltransferase that adds an α-L-fucosyl residue to the O-4 position of the subterminal N-acetylglucosamine of the H or precursor Type 1 chain (Figure 1.5). The type of acceptor molecule, H Type 1 or precursor Type 1, determines the type of Lewis determinant formed, Lewis^b (Le^b) or Lewis^a (Le^a), respectively. Lewis^x and Lewis^y are isomers of Le^a and Le^b respectively, formed on Type 2 structures by the action of a α-3-L-fucosyltransferase. The gene that codes for this enzyme is situated at a site distinct from the *Lewis* locus.

In saliva and other secretions, Le^a and Le^b determinants are carried on the same types of glycoprotein molecules as the ABH determinants (Watkins, 1974).

In 1948, Grubb reported that individuals whose red blood cells typed as Le^a positive and who had only Le^a in their secretions did not express the ABH determinants in their secretions (non-secretors). In contrast, individuals whose red blood cells typed as Le^b positive were secretors of ABH determinants and in addition expressed Le^a and Le^b in their body fluids. Now it is known that red blood cells of secretors express some Le^a, but its quantity is insufficient for detection by haemagglutination using anti-Le^a antibodies. Individuals who do not express any Lewis determinants on their red blood cells (Lewis negative) could be either secretors or non-secretors; and their saliva does not contain Le^a or Le^b. It should be stressed that the presence of Lewis antigens in secretions and on cell surfaces depends on the expression of the *Lewis* gene and not on the *secretor* gene.

Genetic control and interaction with the *Se*, *H* and *ABO* genes

The blood group determinants present on cell surfaces and in secretions are the end products of sequential glycosylation of carbohydrate chains. Several factors

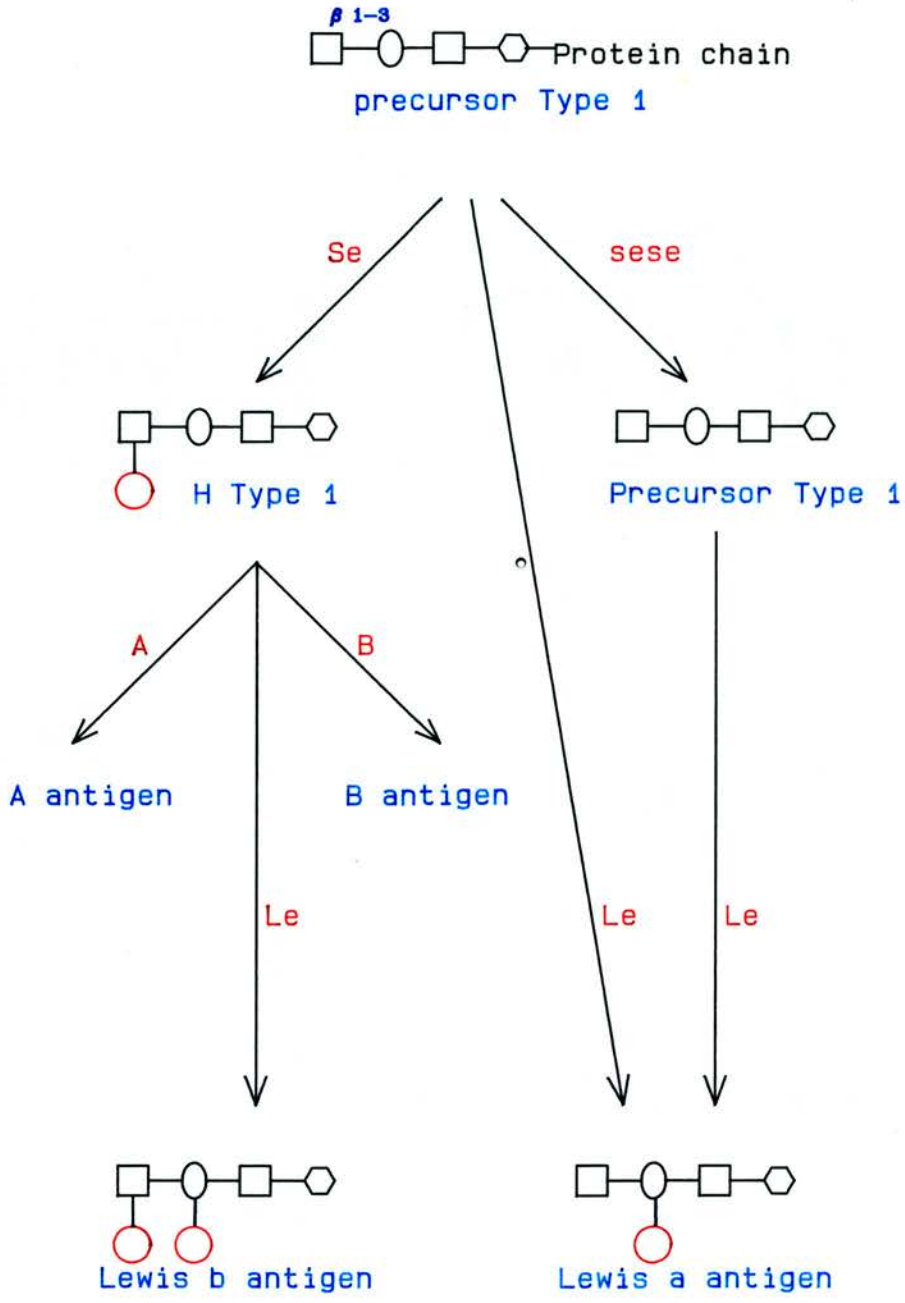

Figure 1.5: The pathway of production of H, B, A and Lewis determinants in secretions. Symbols as in Figures 1.1 and 1.2. The arrows represent the reactions possible in individuals possessing the genes indicated by the letters.

are involved in regulating the expression of the blood group determinants. The first is the glycosyltransferases found within an individual. These depend on the alleles inherited at the blood group loci and the transcription and translation of these genes. The sequence of glycosylation is another factor which influences the blood group repertoire. The action of one enzyme on a precursor chain can result in either production of the acceptor for another enzyme or changing the structure to make it unsuitable as an acceptor for another glycosyltransferase. Since enzymes coded by different genes can compete for the same acceptor, the amount of enzyme and its kinetic properties are important factors influencing the type of determinants produced.

The construction of A and B determinants as part of the cell membrane depends on the action of a fucosyltransferase on Type 2 precursor chains. H Type 2 is the acceptor for the A and B immunodominant monosaccharides; therefore, if H Type 2 is not formed, as in individuals with the Bombay or para-Bombay phenotypes, the A and B determinants cannot be expressed. Once H Type 2 is formed, further glycosylation of this structure is determined by the genes expressed at the ABO locus. Individuals possessing the *A* or *B* alleles, will convert the H determinant to A or B respectively. If both *A* and *B* genes are present, the enzymes coded by these genes will compete for the same acceptor substance (H Type 2); and cells of these individuals (AB phenotype) will possess some molecules carrying the A determinant and some carrying the B determinant. Recently it has been observed that although each blood group antigen carries an average of 2 or 4 determinants, the A and B determinant are not found on the same carrier molecule (Viitala *et al.*, 1986). The rate of expression of each one of these determinants depends on the relative efficiency and quantity of the enzymes involved. The *O* allele does not give rise to a functional product; therefore, individuals inheriting two *O* alleles will express unmodified H Type 2 on

their cell membranes (O phenotype).

In secretions the Type 1 chain is the acceptor substance for the production of blood group determinants. Two fucosyltransferases coded at different genetic loci (*Se* and *Le*) may compete for this structure. When both Se and Le enzymes are expressed, the Type 1 precursor chain is first fucosylated by the Se enzyme to produce H Type 1 determinants. This structure can be further fucosylated by the Le enzyme to form Le^b determinant. If the precursor structure is first utilized by the Le enzyme, the structure formed (the Le^a determinant) is unsuitable as a substrate for the Se enzyme. As a result, both Le^b and Le^a are formed in secretors; the relative amounts found depends on the relative activity and level of the Se and Le enzymes (Watkins *et al.*, 1988). The A and B enzymes also compete with the Le enzyme for the same substrate. Once a determinant has been converted to Le^b, it cannot function as a substrate for the A and B transferases. On the other hand, A and B determinants can be further fucosylated by the Le enzyme to form ALe^b or BLe^b determinants.

In non-secretors, since the fucosyltransferase coded by the *Se* gene is not produced, the only determinant formed is Le^a, providing the *Le* gene is active. In the rare individuals in whom neither *Se* nor *Le* are present, secretions will contain unconverted precursor Type 1.

The genes involved in the expression of the blood group determinants in secretions and on cells and the pathways leading to the production of these determinants are summarised in Figures 1.4 and 1.5.

1.2 Blood Group Antigens and Disease Susceptibility

1.2.1 Introduction

The blood group antigens are a genetically determined characteristic of an individual's cells. The frequency of the genes coding for the enzymes that produce blood group antigens differ between populations. These differences could arise through two mechanisms; genetic drift and natural selection. Genetic drift can be demonstrated by the "founder effect": when a small group of people migrate to an isolated place and proliferate. If the small group differs from the original population with respect to certain blood group gene frequencies, this will result in differences in the frequencies of these genes in the isolated population compared with their population of origin. Natural selection will influence the gene frequencies if there are differences in the fitness of the various blood groups. Most workers now agree that both forces are taking part in establishing the distribution of blood types in the world (Mourant *et al.*, 1978). Since infectious diseases are involved in the second factor, this will be discussed in more detail.

The selection against a blood group is determined by the environment and should result in early mortality or lower fertility of individuals carrying this blood type. If in a particular environment certain diseases are more common or tend to be more severe in people of a certain blood type, the frequency of this blood type should be lower in comparison to other populations where such condition does not exist. An example is the influence of the malaria parasite on the distribution of the Duffy blood group antigen in Africa. This example will be described in the following sections. The forces of natural selection in the case

of bacterial infections had more influence prior to the advent of antibiotics and the use of modern therapy when such infections resulted in high infant mortality.

The first attempt to study an association between blood group antigens and disease susceptibility was performed by Buchanan and Higley (1921) after the variation in blood-group frequencies between populations was reported (Hirszfeld and Hirszfeld, 1919). In their study, Buchanan and Higly determined the ABO blood groups of 2446 patients suffering from various diseases. They found no relationship between blood group and disease, probably because the numbers tested for each disease were too small to yield statistically significant results. Since then, many statistical studies have been done comparing the frequencies of the different blood group systems in people suffering from a particular disease or infection with the frequencies found in the normal population. The association between the ABO blood groups and infectious diseases identified by statistical surveys were reviewed by Blackwell (1989b). An association between diseases and secretion of blood group antigens has also been found in epidemiological studies. Individuals who do not secrete the ABH blood group antigens into their body fluids (non-secretors) were found to be more susceptible to a wide range of diseases compared to secretors of blood group antigens. This thesis is concerned with this association which will be described in section 1.2.3.

Some of the strongest associations between diseases and blood groups have been reported for conditions other than infections: cancer, thrombosis and autoimmune disease (see Mourant *et al.*, 1978). These are beyond the scope of this thesis and will not be included, apart from certain autoimmune diseases for which infectious triggers have been suggested and for which an association with non-secretion of blood group antigens has been observed.

Two main hypotheses have been proposed to explain the differential suscep-

tibility of individuals of a particular blood type to an infectious agent. The first is based on the experiments that have shown that antibodies against ABH antigens cross-react with certain Gram-negative bacteria (Springer *et al.*, 1961). It has been suggested that such cross-reaction could promote rapid phagocytosis in individuals with antibody to that particular blood group antigen (Eichner *et al.*, 1963). These "natural antibodies" might protect individuals from disease when exposed to bacteria expressing the cross-reactive antigen. The second hypothesis is based on experiments that demonstrated the binding of some microorganisms to particular blood group determinants. It has been suggested that blood group structures on the host cell can serve as a binding site for microorganisms, thereby increasing susceptibility of individuals possessing these structures to infections.

Other immune defence mechanisms have been suggested to contribute to the increased susceptibility of non-secretors to infections. These will be described later in this introduction. This thesis examines differential susceptibility of non-secretors to *Neisseria meningitidis* in terms of blood group antigens as receptors to bacteria.

1.2.2 The Duffy blood group antigen, susceptibility to malaria and natural selection

The importance of infectious diseases in natural selection was first recognized by Wells in 1818 who suggested that the genetic distribution of the populations in Africa had been influenced by resistance to local diseases (see Barnwell *et al.*, 1989). To date, the only disease that has been demonstrated to influence population genetics is malaria.

The frequencies of two distinct genes, both related to red blood cells, have been associated with susceptibility to malaria: the gene coding for haemoglobin S and that which codes for the Duffy antigen. Two alleles control the expression of haemoglobin. In most parts of the world haemoglobin A is the normal type found. In some areas, especially in Africa, haemoglobin A is partially replaced by haemoglobin S. Heterozygous individuals expressing one allele for normal haemoglobin (A) and one of the rare allele (S), will have a mixture of both types of haemoglobin; these individuals are healthy. In contrast, individuals homozygous for the S gene will have sickle-cell anaemia and usually die in infancy. Although the S gene is lethal, it is present in Africa at a high and stable frequency compared with other parts of the world. The reason for that is probably the fact that individuals heterozygous for haemoglobin (AS) are more resistant to malaria caused by *Plasmodium falciparum* than normal individuals (AA) (for a review see Mourant, 1983). Cells carrying the S gene have a mechanical distortion that by unknown mechanisms leads to the destruction of the parasite.

The association between the expression of the Duffy blood group antigen and malaria is the only case where the distribution of blood type shows a strong association to the presence of an infectious agent. For many years it has been known that the distribution of the two major phenotypes of the Duffy antigens Fy^a and Fy^b, vary dramatically between white and black populations. In white populations almost all individuals are positive for one or two of the *Duffy* alleles. In contrast, the majority of Negroes in and outside Africa are negative for the two types of Duffy antigen (Sanger *et al.*, 1955).

It is known that African and American Blacks are resistant to malaria infection due to *Plasmodium vivax* which is highly infective to other races. Miller

et al. (1975) were the first to report an association between the Duffy blood type and susceptibility to *P. vivax*. Direct studies on *P. vivax* could not be performed, since the parasites were difficult to obtain and could not be cultured. Instead, *Plasmodium knowlesi*, which *in vivo* invades rhesus monkey erythrocytes and is closely related to *P. vivax*, was used to study the invasion of human red blood cells of different Duffy types. In these experiments, it was found that invasion rates of three Duffy phenotypes (Fy^{a+b-}, Fy^{a-b+} and Fy^{a+b+}) were similar, but almost no invasion was observed for Duffy negative erythrocytes (Fy^{a-b-}). They also reported that once cells were treated with proteolytic enzymes which destroy the Duffy determinant, the cells were resistant to invasion. Malaria merozoites can attach to both Duffy negative and Duffy positive cells, but the invasion process is only possible in Duffy positive cells. On the basis of these observations, they have speculated that at least two receptors are used for attachment of merozoites to red blood cells; the Duffy determinant being the receptor for the internalization phase. This report became more relevant to human malaria when 11 black and 5 white volunteers were experimentally exposed to bites of mosquitos infected with *P. vivax* (Miller *et al.*, 1976). In this study, they found that only the Duffy negatives (5 blacks) were resistant to erythrocyte infection. Only recently, a direct *in vitro* study has been preformed which confirmed previous studies and showed that a Duffy determinant, Fy^6, is a receptor for *P. vivax* merozoites (Barnwell *et al.*, 1989). This structure was reported to be expressed in all human red cells except Fy^{a-b-} (Nichols *et al.*, 1987). The Fy^6 determinant was found to be the receptor for two species of malaria: *P. vivax* and *P. knowlesi*. Barnwell *et al.* (1989) also showed that *Plasmodium falciparum* merozoites use a different receptor not related to the Fy^6 determinant.

Although *P. vivax* infection rarely causes death in Western populations, it

may decrease survival in African children who are malnourished or who have other endemic diseases (Miller *et al.*, 1976). Since erythrocytes are the site of reproduction of malaria parasites, it is expected that genes which increase resistance of red blood cells to invasion by the parasite will occur in higher frequency in areas hyperendemic for malaria.

In his book 'Blood Relations', Mourant (1983) suggests a two stage evolutionary process, which selects for the distribution of the genes *A*, *S* and *Duffy*, and the type of malaria found in Africa. At first, *P. vivax* was endemic in Africa as it still is in other parts of the world. A mutation in the *Duffy* locus gave rise to a new gene which does not code for the two common types of Duffy determinants, Fy^a and Fy^b. This gene spread in the population and gave rise to a few homozygotes who were completely resistant to malaria and, therefore, had a selective advantage. As a result of natural selection, the gene spread until the majority of the population did not express the Duffy antigen used as a receptor for invasion by *P. vivax* (probably Fy^6 as recently reported). It is possible that for some time Africa was almost free of malaria until *P. vivax* underwent a mutation which converted it into a form that could invade Duffy negative red blood cells. This mutation created a new species, *P. falciparum*, which replaced *P. vivax* in Africa, also as a result of natural selection.

The mutation of the malaria parasite created a much more virulent disease agent than the original parasite and led to a second genetic change in the African population. This time a new type of haemoglobin evolved, haemoglobin S, which provided complete resistance to *P. falciparum*. This mutation could not completely replace the original type of haemoglobin (haemoglobin A), since individuals who are homozygous for this allele die at a young age. In the heterozygotes, it provides resistant to malaria and almost no other effects.

The evolutionary process described can explain the high frequency of Duffy negative erythrocytes and haemoglobin S in Negroes; and the existence of *P. falciparum* as the main type of malaria causing agent in tropical Africa.

1.2.3 Secretor status and disease susceptibility

Several epidemiological studies have reported an association between the ability to secrete blood group antigens into body fluids and various diseases. In these studies, individuals in patient populations suffering from a specific disease were defined as secretors or non-secretors on the basis of the presence of the ABH antigens in their saliva. The proportions of secretors and non-secretors of the patient group were compared to those of a control population. In the studies that have shown an association, non-secretors of the ABH determinants were found to be more susceptible to the disease. The diseases that have shown an association with secretor status can be divided into two groups; infectious diseases (Table 1.1) and autoimmune diseases (Table 1.2). A number of different bacterial and fungal infections of mucosal surfaces have been associated with non-secretion. The increased susceptibility of non-secretors to infection due to *Neisseria meningitidis* is the topic of this thesis and it will be described in detail in the next section. Among autoimmune diseases, only conditions that have a proposed infectious trigger were found to be associated with secretor status (see Table 1.2). From these observations, it was suggested that the increased susceptibility of non-secretors to autoimmune diseases is secondary to their being more susceptible to the infectious agent that triggers the autoimmune condition. An example of this is the higher proportion of non-secretors among patients with rheumatic fever and rheumatic heart disease (reviewed by Haverkorn and Goslings, 1969). These conditions are known to occur following streptococcal

Table 1.1: Infections associated with non-secretion of blood group antigens

Infectious agent	Reference
Urinary tract	
E. coli	Kinane *et al.*, 1982
	Blackwell *et al.*, 1987b
Gastointestinal tract	
V. cholerae	Chaudhuri and Das Adhikary, 1978
Genital tract	
C. albicans	Thom *et al.*, 1989
Respiratory tract	
Strep. pyogenes	Haverkorn and Goslings, 1969
N. meningitidis	Blackwell *et al.*, 1986a
N. meningitidis (carriage)	Blackwell *et al.*, 1990
Strep. pneumoniae	Blackwell *et al.*, 1986a
H. influenzae	Blackwell *et al.*, 1986b
Oral cavity	
C. albicans	Thom *et al.*, 1989
C. albicans (carriage)	Burford-Mason *et al.*, 1988
Caries	Arneberg *et al.*, 1976
	Holbrook and Blackwell, 1989

Table 1.2: Autoimmune diseases associated with non-secretion of blood group antigens and the microorganisms that have been proposed to trigger the disease

Disease	Reference	Infectious trigger	Reference
Rheumatic disease			
Rheumatic fever	Haverkorn and Goslings, 1969	*Streptococcus pyogenes*	Kaplan and Meyerserian, 1962
Seronegative arthropathies			
Ankylosing spondylitis	Shinebaum *et al.*, 1987	*Klebsiella pneumoniae*	Geczy *et al.*, 1985
Reactive arthritis	Shinebaum *et al.*, 1987	*Shigella flexneri*	Simon *et al.*, 1981
		Yersinia enterocolitica	Van Bohemen *et al.*, 1984
Endocrine diseases			
Insulin dependent diabetes	Blackwell *et al.*, 1987(a)	Coxsackie virus	Barret-Connor, 1985
		Upper respiratory tract infection	Morris, 1989
Graves' disease	Collier *et al.*, 1988	*Yersinia enterocolitica*	Weiss *et al.*, 1983 Heyma *et al.*,1986

infections where more non-secretors are found among the patients and among carriers of the bacteria than in the control population (Haverkorn and Goslings, 1968).

Recently, secretors have been found to be more susceptible to several viral infections (M. Raza, personal communication) . This study is the first to suggest an association between secretor status and viral infection and it is the first condition in which secretors are over-represented.

The secretion of the A, B, H antigens into saliva and other body fluids, is controlled by the *Secretor* gene which is inherited in a Mendelian dominant fashion. While 25% of the population are expected to be homozygous for the recessive gene (non-secretors phenotype), the proportion of non-secretors varies in different parts of the world. As mentioned before, the forces that are thought to play a role in determining the gene frequencies of a blood group system are genetic drift and natural selection. The selective pressures on the *Se* gene are not clear. The epidemiological data suggests selection against the non-secretor phenotype, since non-secretors are more susceptible to bacterial and fungal infections. On the other hand, a selection against the secretor gene was proposed for haemolytic disease of the newborn due to ABO incompatibility (for a review see Mourant, 1982). It was suggested that infants with the secretor phenotype are more susceptible to this condition; however, the role of secretor status of the infant in the pathogenesis of this condition has not been conclusively determined. The frequency of the *Se* gene in many parts of the world is as expected by its mode of inheritance. The variation in the frequencies of this gene in isolated populations is more likely to be due to founder effect than natural selection (Eriksson *et al.*, 1986). Examples of variations in *Se* gene frequency are shown in a study done in northern populations (Eriksson *et al.*, 1986). In

this study Icelanders were found to have one of the highest proportion of non-secretors (up to 35.94%); and the frequency of non-secretors among Finnish Lapp populations was found to be the lowest reported in Europeans (2.2%). Variations in the *Se* gene frequency are also seen between communities in the same country; for example the proportion of non-secretors in Plymouth and Stonehouse compared to blood donors in South-west England (Phipps and Perry, 1989; Blackwell and Weir, 1990) (see Table 1.3). These variations emphasize the importance of testing the proportion of secretors and non-secretors in the local population, when performing an epidemiological study.

1.2.4 Secretor status and susceptibility to meningococci

The association between the expression of the *secretor* gene and infection by *Neisseria meningitidis* was reported in several studies. Three studies in different parts of the world have found higher proportions of non-secretors among patients with meningococcal disease compared to the proportion of non-secretors in the local population (Blackwell *et al.*, 1988). The results observed in these studies also demonstrate variations in the proportion of non-secretors in different populations and hence the need for a local control population (Table 1.4).

In 1986, a large scale community survey was undertaken in the town of Stonehouse, Gloucestershire, during an outbreak of meningococcal disease (Blackwell *et al.*, 1989b). This survey has shown a higher proportion of non-secretors among patients with meningococcal disease compared with their proportion in the local control population (see Table 1.4). In addition, in this study the proportion of non-secretors in Stonehouse was found to be significantly higher than

Table 1.3: Geographical clustering of non-secretors in relation to outbreaks of meningococcal disease

	Non-secretors (%)		
	outbreak	surroundings	P-value
Study 1:			
Airdrie	37.6		
Glasgow		26.2	<0.0005
Edinburgh		26.2	<0.005
Study 2:			
Stonehouse	32.7		
South-west England		23.4	<0.005
Study 3:			
Plymouth	37.4		
South-west England		23.4	<0.0005

P values were obtained by comparing the proportion of non-secretors in the outbreak town with the proportion in its surrounding area.

Table 1.4: Proportion of non-secretors among patients with invasive infection due to *N. meningitidis* and local control

	Total	Non-secretors(%)	P-value
Scotland			
Controls	334	26.6	<0.005
Patients	26	69	
Stonehouse			
Controls	227	32.7	NS
Patients	13	54	
Iceland			
Controls	228	41.2	<0.05
Patients	98	54	
Nigeria			
Controls	186	49.5	<0.01
Patients	42	73.3	

P values were obtained by comparing the proportion of non-secretors among patients with the proportion of non-secretors among local control population.

the proportion of non-secretors among blood donors in the South-west region of England (see Table 1.3). These studies indicate that non-secretors are at a greater risk for meningococcal disease and suggest that prolonged outbreaks of meningococcal disease appear in areas with a high proportion of non-secretors.

In a subsequent study, the proportion of non-secretors was examined at Airdrie Academy, Lanarkshire, where a short localized outbreak of meningococcal disease occurred (Blackwell *et al.*, 1990). Pattern similar to that found previously was observed. The proportion of non-secretors among pupils and staff in Airdrie was found to be significantly higher than the proportion of non-secretors among blood donors in Edinburgh and Glasgow (See Table 1.3). In addition, a higher proportion of non-secretors was found among carriers of *N. meningitidis* (47%) compared with the control population (32%) (Blackwell *et al.*, 1990). These results support the hypothesis that one of the factors contributing to the susceptibility of non-secretors to meningococcal disease is their increased risk for carriage of the bacteria. The interactions between the host and the bacteria underlying these observations are not understood. Knowledge of meningococcal pathogenesis and factors influencing susceptibility of an individual or population to this disease are important in development of means to prevent meningococcal infections. As described in the next section, methods for the prevention of meningococcal disease are limited at present.

1.3 Meningococcal infection: biology and prevention

In the United Kingdom at least one child in every 1000 develops acute bacterial meningitis by the age of 10. In developing countries, the incidence of bacterial meningitis is probably higher than that recorded in the industrialized West. In areas of Africa, where meningococcal disease occurs frequently, acute bacterial meningitis in childhood may reach a figure as high as 1:100.

Most cases of invasive meningococcal disease are due to encapsulated strains of the bacterium. Isolates of *N. meningitidis* can be serogrouped on the basis of the antigenic structure of their capsular polysaccharides. These serogroups can be further divided into serotypes on the basis of their outer membrane proteins. Eight main serogroups of meningococci are recognized (A, B, C, X, Y, Z, 29e and W135). Most clinical infections are caused by meningococci belonging to serogroups A, B, or C.

Meningococcal infection is endemic in most Western industrialized countries, where only modest variations in incidence of meningococcal disease are observed from year to year. In these parts of the world, geographical clustering of cases is frequently seen. In epidemics, *N. meningitidis* strains belonging to group B or C account for most cases of disease. Acute bacterial meningitis can occur at any age in previously healthy individuals; however, the disease is seen most frequently in young children and the age distribution may vary depending on the strain and the population effected. Household clustering of cases of meningococcal disease is frequent, especially during epidemics (Greenwood *et al.*, 1979). The risk of infection is up to 1:30 in siblings who sleep in the same room as

patients.

The bacterium is spread mainly by respiratory droplets and transmission is enhanced by overcrowding. Once inhaled, the bacteria may establish themselves in the upper respiratory tract; and, in most individuals, infection is restricted to this site. The prevalence of colonization in non-epidemic periods is between 5 and 10% (Greenfield *et al.*, 1971).

Meningitis can follow spread of bacteria from the respiratory tract through the blood. Unless the skull has been fractured, direct spread from the nasopharynx to the meninges is rare. If septicaemia occurs, it usually does so within a short period after colonization. Infection of the meninges can be prevented at two stages: at colonization or after invasion of the blood. At the colonization stage, mucosal defence mechanisms such as secretory IgA might play an important role. In the blood, bactericidal substances provide a second protective barrier which must be overcome before the brain is reached. Serum bactericidal substances include specific antibodies against the capsular polysaccharides and membrane proteins. Their importance in preventing meningitis is demonstrated by the correlation found between resistance to disease and high levels of serum antibodies to the bacteria. Formation of specific serum antibodies can follow asymptomatic nasopharyngeal carriage or infection with harmless commensal bacteria that share antigenic determinants with the pathogen. Thus carriage of harmless bacteria such as *N. lactamica* can induce protective antibodies against meningococci. On the other hand, infection with cross-reactive organisms may induce IgA antibodies that have been reported to block bactericidal activity of IgG and IgM (Griffiss, 1975).

Because nasopharyngeal carriage is much more frequent than meningococcal disease, carriers rather than patients are the usual source of new meningococcal

infection. The ratio of carriage to cases varies from 100:1 during an epidemic to 1000:1 during non-epidemic periods. As carriage may persist for months, carriers can act as a persistent source of infection. Possible ways of decreasing the spread of meningococcal meningitis include treatment of susceptible hosts or reduction of the pool of infection by reducing carriage rate. The two main approaches are chemoprophylaxis and immunization.

Sulphonamides were effectively used for over 20 years to eradicate meningococcal carriage and disease in military recruits. In 1963, an outbreak of meningococcal meningitis due to group B sulphadiazine resistant meningococci occurred in San Diego (Millar *et al.*, 1963). Since then, an increased proportion of sulphonamide resistant strains have been reported. The recent epidemics in Stonehouse and Airdrie were due to sulphonamide-resistant strains (Cartwright *et al.*, 1987; Blackwell *et al.*, 1990). In the search for other antimicrobial agents, penicillin G, erythromycin, oxytetracycline and ampicillin were found to be ineffective in reducing colonization by the sulphonamide-resistant strains (Dowd *et al.*, 1966). Later, rifampicin was found to be efficient in reducing carriage, but studies have shown that resistance developed rapidly among staphylococci, enteric bacteria and meningococci (for review see Broome, 1986). At present, minocyclin is widely used for prophylaxis and is recommended for use for household contacts of patients with meningococcal meningitis. The development of minocyclin resistance has not been a problem and a very low proportion of resistant strains (0.15%) has been reported (Band *et al.*, 1983).

Another method used for prevention of meningococcal disease is vaccination with meningococcal polysaccharides (group A, C, Y and W135). The efficacy of these vaccines for reducing carriage is questionable. Initially, the use of group C polysaccharide was found to reduce carriage rate by 30–50% (Gotschilch *et*

al., 1969). More recently, a vaccination trial in Africa showed that the use of group A polysaccharide had no effect on the carriage rate (Blakebrough *et al.*, 1983). Vaccination with these polysaccharides is used mainly for military personnel. Group C vaccine is not effective in children under the age of 2 years and a vaccine against group B strains is not available. Since serogroups B and C meningococci are the main causes of meningitis and one of the most susceptible populations are young children, vaccination of civilians is not feasible. Many studies are now directed at developing meningococcal vaccines that will be safe, inexpensive, easily administered, immunogenic in infants and provide prolonged immunity (for review see Griffiss *et al.*, 1987). Determination of the host parasite interactions that enhance susceptibility of individuals to the disease might facilitate this goal and identify the population that should be immunized.

1.4 Hypotheses proposed for the association between secretor status and disease

1.4.1 Secretor status and immunoglobulin level

An association between the level of immunoglobulins and secretor status has been studied in an attempt to explain the increased susceptibility of non-secretors to infections. The first report of differences in antibody levels between secretors and non-secretors was an increased level of isoantibody in secretors compared with non-secretors (see Grundbacher and Shreffler, 1970). In a subsequent study, Grundbacher and Shreffler (1970) confirmed this observation by demonstrating higher levels of anti-B in secretors compared with non-secretors. Secretors were found to have a significantly higher level of total serum IgG compared with

non-secretors. This higher total IgG level has been suggested to account for the increase in isoantibody level. Similar results, where secretors were found to possess higher levels of immunoglobulins, have been reported for total salivary IgA (Waissbluth and Langman, 1971) and serum IgA (Grundbacher, 1972). Another study reported higher levels, though not statistically significant, of serum IgG and IgM in secretors compared with non-secretors (Waissbluth and Langman, 1971). As suggested by the authors, the differences were probably not statistically significant because of the small numbers tested .

The lower immunoglobulin levels found in non-secretors compared with secretors were used to explain the increased susceptibility of non-secretors to rheumatic fever and rheumatic heart disease (Glynn *et al.*, 1956; Clarke *et al.*, 1960). These two conditions follow a streptococcal infection which has been shown to be associated with non-secretion of blood group antigens. The initial infection could be influenced by the ability of an individual to generate an immune response to the bacteria. Therefore, lower level of immunoglobulins found in non-secretors compared with secretors might result in increased susceptibility to streptococcal infection. This increased infection rate will predispose the non-secretors to rheumatic fever and rheumatic heart disease.

In a later study on the association between secretor status and immunoglobulin levels, total serum IgA and IgG were compared by a quantitative radial immunodiffusion assay in women with recurrent urinary tract infections (UTI) (Blackwell *et al.*, 1987b). The women in this study were divided into two groups according to the length of time they had suffered from these infections. In both groups, newly referred and long term sufferers, there was an increased proportion of non-secretors among the patients compared with local controls. In the group of patients recently referred to the clinic, no difference was observed in the

amounts of serum IgA or IgG between secretors and non-secretors. In contrast, among patients monitored by the clinic for the last 20 years, non-secretors were found to have higher levels of total serum IgA and IgG.

In this report, unlike previous studies, the patients suffered from recurrent infections. Repeated episodes of a particular infection will result in increased levels of immunoglobulins specific for the pathogen. If the increase in specific immunoglobulin (not measured in this study) is reflected in the total immunoglobulin level, then higher levels of total antibodies might simply reflect an increased number of episodes. This possibility is supported by the higher level of immunoglobulins found in the patients suffering from recurrent UTI for the last 20 years, compared with patients recently referred to the clinic (Blackwell *et al.*, 1987b).

It is possible that the lower immunoglobulin levels in healthy non-secretors compared with secretors reported in earlier studies, predisposes non-secretors to initial infections. In this case, non-secretors will suffer from more episodes of colonization than secretors since their immunoglobulin levels in the healthy state are lower than secretors. Repeated episodes of infection will result in high levels of immunoglobulins, as reported for women suffering infections for many years (Blackwell *et al.*, 1987b). Non-secretors who suffered from infections for a shorter period show the same immunoglobulin levels as secretors, since the higher immunoglobulin levels reported for healthy secretors is balanced by the increased episodes of colonization in non-secretors. The high level of antibodies in non-secretors became apparent only after a long period of recurrent infections.

A later study comparing total salivary IgA levels in secretors and non-secretors among carriers of *N. meningitidis* and in non-carriers (Blackwell *et al.*, 1989a) supports this hypothesis. In this study, among carriers of the bac-

teria, no difference was found in total salivary IgA levels between secretors and non-secretors. However, carriers of the bacteria had higher levels of salivary IgA than non-carriers. This suggests two explanations: the specific response is reflected in the total immunoglobulin levels; or, bacterial components cause polyclonal B cell activation. In contrast, among non-carriers a higher level of serum IgA was found in secretors compared with non-secretors.

The class of antibody produced, not just the quantity of antibodies, is an important factor in protecting against infections. As suggested by Blackwell *et al.* (1989b) high levels of IgA, specific for the capsular polysaccharide in serum of non-secretors, might compete with complement fixing isotypes (IgG and IgM) for binding sites on the surface of the pathogen. The ability of IgA to block bactericidal activity of meningococcal antisera has been demonstrated (Griffiss, 1975).

Conflicting results have been reported for the differences in immunoglobulin levels between secretors and non-secretors. No difference in total serum or salivary concentration of IgA between secretors and non-secretors was reported among patients with spondyloarthropathies (Shinebaum *et al.*, 1987). This study reported a higher proportion of non-secretors among these patients which does not seem to be the result of a deficiency or overproduction of IgA.

The importance of any difference in immunoglobulin level in the increased susceptibility of non-secretors to infections is not clear. It has been suggested that the concentrations of immunoglobulins differ between secretors and non-secretors depending on their state of health and infection. The level of immunoglobulins and their isotype might play an important role in the increased susceptibility of non-secretors to infections, or conditions triggered by infections. Further investigations of the specific immune response of secretors and

non-secretors to pathogens and its effect on the total immunoglobulins levels are required.

1.4.2 Complement levels and secretor status

The gene that codes for the third component of the complement system (*C3*) is located on chromosome 19 and was found to be closely linked to the *secretor* gene (Elberg *et al.*, 1983). Since complement is an important component of the immune system, C3 has been proposed as a factor which might play a role in the association between secretor status and disease.

The third component of complement occupies a critical position in the activation of the complement system. Many of the biological functions of this system are elaborated with the activation of C3. Activation of C3 by the classical or alternative pathways results in generation of an anaphylatoxin that stimulates an inflammatory response. The larger fragment (C3b) can bind to cell surfaces such as bacteria and activate the cascade from C5 to C9 resulting in cell lysis. The binding of C3b to a particle also acts as an opsonin mediating phagocytosis; immune complexes are bound by macrophages, monocytes, polymorphonuclear leucocytes and B lymphocytes by means of receptors specific to C3b and its breakdown products.

Complement levels have been suggested to play a role in the association found between non-secretors and some disease conditions (Blackwell 1989b). In the Stonehouse study C3c levels among non-carriers of *N. meningitidis* were determined by quantitative radial immunodiffusion. The level of C3c was found to be slightly lower in non-secretors compared with secretors. In 8 non-carrier individuals, C3c levels were found to be below the normal range; 7 of these were

non-secretors (Blackwell, 1989b). Among patients with diabetes, lower levels of C3c were found in patients with insulin dependent diabetes mellitus (IDDM) but not among those with non-insulin dependent diabetes mellitus (NIDDM) (Charlesworth *et al.*, 1987). Non-secretors of blood group antigens were found to be over-represented among patients with IDDM but not among NIDDM (Blackwell *et al.*, 1987a). These two observations triggered a third study on patients with IDDM, in which lower levels of C3c were found among non-secretors compared with secretors (Blackwell *et al.*, 1988). Development of IDDM is not linked only to genetic factors, environmental factors such as infectious agents have been suggested to play a role in the aetiology of the disease (see Table 1.2). It is possible that C3 levels could influence the occurrence of the infectious state which might predispose certain individuals to develop IDDM. The association between secretor state and C3 levels is not well established and the biological significance of differences in complement levels are not known.

A factor which was not considered in the association between secretor status and C3 is the possible association with the two main variants of C3. The third complement component exhibits genetic polymorphism (reviewed by Alper, 1986). The genetic variants of the protein are inherited as an autosomal codominant trait. Several variants have been found and characterized by agarose gel electrophoresis; but only two, C3S (slow) and C3F (fast) occur frequently. In most populations the sum of the gene frequencies of *C3S* and *C3F* are 99%, where the gene frequency of *C3S* ranges between 77–99% (Raum *et al.*, 1980).

The biological significance of the C3 polymorphism is still unknown. No difference between the two variants have been found with regard to immunochemically measured quantities, the stability, the molecular size or the haemolytic capacity of sera of different C3 phenotypes (reviewed by Bronnestam, 1973).

Cytolysis is only one of the activities of C3. This molecule is also involved in immune adherence and promotion of phagocytosis. In only one study has a difference between the two variants regarding these functions been reported (Arvilommi, 1974). In this study, C3F was found to have an enhanced capacity to bind to complement receptors on human mononuclear cells.

Several studies have reported a significant increase of the *C3F* allele among patients with various diseases (Bronnestam, 1973; Elmgreen *et al.*, 1984; Jans and Sorensen, 1980; Regueiro and Arnaiz-Villena, 1984; Schiotz *et al.*, 1978; Srivastava and Srivastava, 1985;). A relationship was also found between C3 polymorphism and antibody titre (Bronnestam and Cedergren, 1973). In this study, C3S was associated with high titres of immune antibodies to A and B blood group antigens in mothers with ABO incompatible infants. The mechanism behind this observation is not known but it was suggested that C3 variants might influence antibody production (Bronnestam and Cedergren, 1973). Although functional differences between the two variants are not fully understood, such differences might contribute to the increased susceptibility of patients who inherited the *C3F* gene. Since the *Se* and the *C3* genes are linked, it is possible that the inheritance of *se* along with the *C3F* allele could account for the increased susceptibility of non-secretors to diseases.

1.4.3 Blood group antigens as receptors for microorganisms

Blood group antigens are found on the surface of many tissues, including the surface of epithelial cells which are often the site of colonization for microorganisms. The serologically defined carbohydrate structures that form the blood

group determinants are present as a constituent of various macromolecules. Many macromolecules in secretions are glycosylated and the expression of certain blood group determinants in this situation will be under the control of the *secretor* gene. Some of the blood group antigen structures found on the surface of cells are not made endogenously; they are adsorbed from secretions onto the cell surface. Hence, the *secretor* gene influences the distribution and presence of some carbohydrate structures on the surface of epithelial cells and in secretions.

The cell surface is abundant in carbohydrate molecules; and, carbohydrate structures have been postulated to be one of the most probable attachment site for microorganisms (Lark, 1986). The role of carbohydrate structures in specific attachment of viruses and bacteria to cells has been extensively studied. The work of Burnet *et al.* in the 1940's described an enzyme activity, "receptor destroying enzyme", responsible for specific inhibition of influenza virus binding to the cell surface (See Burnet and Stone, 1947). Later the substance being split off was identified as *N*-acetyl-neuraminic acid and at about the same time it was shown that several bacterial species specifically bind to mannose (for review see Bock *et al.*, 1988). About 15 years later the *N*-acetyl-neuraminic acid containing glycolipid, the ganglioside GM1, was described as the specific receptor for cholera toxin. Binding to this cell surface molecule is a crucial event for the subsequent effects of the toxin (Eidels *et al.*, 1983). Brain gangliosides in a liposome model have been shown to be the binding site for Sendai virus (Haywood, 1974).

Blood group antigens are expressed at a very high density on the surface of the cells. They are widely distributed in the body and show variation in expression between tissues, individuals and species. If blood group antigens act as receptors for microorganisms, then tissue tropisms, individual or species sus-

ceptibility to infection can in part be explained at the receptor level. Although blood group antigens are a potential binding site for microorganisms, there is very little evidence for such a role. The Duffy antigen is known to be the receptor for *Plasmodium knowlesi* (Miller *et al.*, 1975). In 1980, two independent groups (Kallenius *et al.*, 1980; Leffler and Svanborg Eden, 1980) demonstrated specific binding of *E. coli* to Galα1–4Gal containing molecules. These structures are part of the P blood group antigen and are found on cells of the urogenital tract where they play a role in the colonization of *E. coli* strains expressing P fimbria. The Anton antigen has been reported to be the receptor for *Haemophilus influenzae* on oropharyngeal epithelial cells (Van Alphen *et al.*, 1986).

As just described, carbohydrate determinants have been shown to be receptors for microorganisms. Differences between secretors and non-secretors in carbohydrate structures present on the surface of their epithelial cells could influence their susceptibility to colonization by pathogens.

1.5 Aims of this study

Epidemiological studies indicate that non-secretion of blood group antigens is associated with increased susceptibility to several infections and autoimmune conditions. The aim of this study is to examine host-parasite interactions contributing to the increased susceptibility of non-secretors to meningococcal disease and carriage of *N. meningitidis*.

A number of factors might be involved in the increased susceptibility of non-secretors. At present, very few differences have been described between secretors and non-secretors of blood group antigens. Among those that have

been reported, the only ones that can explain the increased susceptibility of non-secretors are: immunoglobulin levels, complement levels and blood group antigen expression. The only factor that is strongly associated with secretor status, is well defined and known to be directly influenced by the *secretor* gene is the expression of several blood group antigens. The differences in the expression of blood group antigens between secretors and non-secretors are summarised in Table 1.5. On the basis of these differences, two hypotheses have been proposed to explain the increased susceptibility of non-secretors to infections (Blackwell, 1989a,b). Both suggest blood group determinants might be receptors for adhesins on the surface of microorganisms.

The first suggests that ABH or Lewis^b antigens in the body fluids of secretors can bind to lectin-like adhesins on the surface of microorganisms and block their attachment to epithelial cells. In the case of *N. meningitidis*, disease and carriage of the bacteria were not associated with specific ABO blood group but with the ability of the individual to secrete the antigens. This suggests that the A and B determinants are not involved in the lower prevalence of secretors among patients with meningococcal disease. The presence of Lewis^b is dependent on the expression of the *secretor* gene, and is found only in the secretions and on the cells of secretors. To suggest this determinant is a receptor that could block the attachment of the bacteria to secretor's cells implies that the same receptor is an attachment site on the cell surface. In this case the attachment of the bacteria to Lewis^b antigen will not explain the increased susceptibility of non-secretors to carriage and disease since this antigen is not expressed on their cells. Since the *H* gene is expressed in almost all people, the H determinant is found on the cells of most individuals independent of their blood type and secretor status. This determinant is found only in the secretions of secretors, independent of their blood type, where its expression is dependent on the presence of the *secretor*

Table 1.5: Blood group antigens found in secretors and non-secretors

Antigen	Secretors		Non-secretors	
	Cells	Secretions	Cells	Secretions
H (A/B)*	+	+	+	-
Lewis^{a} **	+/-	+/-	+	+
Lewis^{b} **	+	+	-	-

\+ Present
\- Absent
+/- Present in small amounts

* Exact structure depends on the genotype
** Dependent on expression of the Lewis gene

gene. If the H determinant is one of the receptors for bacterial attachment on epithelial cells, the presence of this antigen in secretions of secretors might reduce the density of colonization by interfering with attachment. This model could explain the lower prevalence of infection and carriage among secretors.

The second hypothesis suggests that the Lewis^a determinant which is found predominantly in non-secretors is a receptor for bacteria on the surface of cells. There are two pieces of evidence which suggest that the Lewis^a determinant is a receptor for bacterial attachment. Lomberg *et al.* (1986) found that the binding of uropathogenic strains of *E. coli* to cells from non-secretors was higher compared to the binding to cells from secretors, irrespective of their P and ABH blood groups. They suggested that the addition of A, B or H determinants under the control of the *Se* gene to the epithelial glycoconjugates could block the receptor for the bacteria by modifying the surrounding molecules or the receptors themselves. Another possibility, which they did not consider, is that the bacteria bind to the Lewis^a determinant which is the only structure known to be present on non-secretors' cells in higher amounts than on secretors' cells (see section 1.1.3). In another study the binding of *Candida* blastospores to epithelial cells from secretors and non-secretors was investigated. The attachment of the blastospores, from one of the two *Candida* strains tested, to cells from non-secretors was blocked by anti-Lewis^a. Pre-treatment of the epithelial cells with anti-Le^b did not decrease binding (May *et al.*, 1989). It was concluded that the Le^a determinant on the surface of epithelial cells might be one of the receptors for binding blastospores of some strains of *Candida.* This observation might explain the epidemiological studies which have found an increase in the proportion of non-secretors among patients and carriers of *Candida* species (Blackwell *et al.*, 1989b, Thom *et al.*, 1989).

The aim of this study was to investigate the role of blood group antigens as receptors for *N. meningitidis* in the context of the two hypotheses suggested above. Assays were developed to quantitate H and Le^a determinants on cells and/or in secretions and to measure bacterial attachment to epithelial cells. The ability of the bacteria to bind Le^a and H from solution was investigated. Once the determinant that acts as a receptor on the cell is identified, this can be used to examine the structure on the bacteria which interacts with the receptor. Identification and isolation of the bacterial adhesin might be used as an approach for the development of preventive strategies.

Chapter 2

General materials and methods

2.1 Buffers

All chemicals were analytical grade obtained from BDH Chemicals Ltd. (Poole, Dorset, UK) or Sigma (Poole, Dorset, UK).

2.1.1 1M Carbonate-bicarbonate buffer (pH 8.9)

Stock solutions of 1M sodium carbonate and 1M sodium bicarbonate were prepared. The buffers were mixed in the proportion 1:9 (v/v) adjusted to pH 8.9 with one of the stock solutions (carbonate raises pH, bicarbonate lowers pH). The bicarbonate solution was prepared fresh for each experiment.

2.1.2 Dulbecco's phosphate-buffered saline , solution A (DPBS)

This buffer was composed of the following: 136 mM sodium chloride; 2.6 mM potassium chloride; 8.1 mM sodium hydrogen phosphate; 1.47 mM potassium dihydrogen phosphate; 0.2% aqueous phenol red (pH 7.3).

2.1.3 Dulbecco's phosphate-buffered saline (DPBS+B)

This buffer was prepared by adding 0.9 mM Ca^{2+} and 0.4 mM Mg^{2+} ions to Dulbecco's phosphate-buffered saline.

2.1.4 Phosphate-buffered saline (PBS) (pH 7.2)

PBS contained 0.01 M phosphate buffer pH 7.2 with 0.15 M NaCl.

2.1.5 Phosphate citrate buffer (PCB) (pH 5)

PCB contained 0.1 M sodium hydrogen phosphate and 0.1 M citric acid, the pH adjusted to pH 5.

2.2 Subjects

Subjects used in this study were healthy staff and students in the Department of Bacteriology, University of Edinburgh. The ABO blood groups of the donors were determined from heparinized blood samples by slide agglutination with monoclonal antibodies (Scottish National Blood Transfusion Service). The secretor-state of individuals was determined by the haemagglutination inhibition assay with saliva (Mollison, 1983).

2.3 Collection of epithelial cells

Buccal epithelial cells (BEC) from healthy individuals were collected by rubbing the inside of the cheeks with cotton swabs. Nasopharyngeal cell (NPC) and oropharyngeal cells (OPC) were collected from one secretor and one non-secretor with cotton swabs. To remove the cells, swabs were rinsed in 20 ml

DPBS+B. Cells were washed twice in DPBS+B (300 g for 10 min) and their concentration adjusted to $2.5x10^5$ cells/ml after determination of the number of cells microscopically in a haemocytometer.

2.4 Collection of saliva

Unstimulated saliva was collected from healthy individuals, and the samples were centrifuged at 1000 g for 20 min. The supernatant was collected, boiled for 20 min, recentrifuged and the supernatant stored at –20°C until used.

2.5 Bacteria

Serogroupable isolates were obtained from spinal fluid or blood of patients with meningococcal disease. The non-groupable isolate was obtained from a carrier.

2.5.1 Culture media: Modified New York City medium (MNYC)

MNYC medium (Young, 1978) composed of Difco Gonococcal base enriched with 10% horse blood lysed with 0.5% Saponin, 2.5% yeast dialysate, 0.1% glucose, lincomycin (1.0 μg/ml), colistin (6 μg/ml), amphotericin B (1.0 μg/ml) and trimethoprim lactate (6.5 μg/ml).

2.5.2 Incubation conditions

Standard incubation conditions, unless otherwise stated, were overnight at 37°C in an aerobic CO_2-enriched (10%) humidified atmosphere.

2.5.3 Storage of bacteria

All isolated were stored lyophilised at room temperature.

2.5.4 Standardization of bacterial concentration

Bacterial concentration was calibrated by comparison of numbers detected by counting chamber (Thoma) with spectrophotometric reading at 541 nm ($2x10^8$ bacteria/ml=0.35 O.D.).

2.6 Solutions for enzyme linked immunosorbent assay (ELISA)

2.6.1 Coating buffer

Coating buffer was composed of 15 mM sodium carbonate, 35 mM sodium bicarbonate and 3 mM sodium azide (pH 9.6).

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2.6.2 Washing buffer

Washing buffer was prepared by adding bovine serum albumin (BSA) (1% w/v) and Tween-20 (0.05% v/v) to 0.01 M PBS (pH 7.2).

2.6.3 Blocking buffer

Blocking buffer was composed of 0.01 M PBS containing 1% (w/v) BSA (pH 7.2).

2.6.4 Substrate solution

The substrate solution used in the colour reaction to detect horseradish peroxidase contains 40 mg *O*-phenylenediamine in 100 ml of 0.1 M phosphate citrate buffer (pH 5) activated immediately before used by adding 40 μl H_2O_2 (30% v/v).

2.7 Analysis of cells by flow cytometry

Analysis was done on an EPICS 'C' flow cytometer (Coulter Electronics, Luton, UK) equipped with a 5 watt laser using a power output of 300 mw at 488 nm. The cells were selected from a display of forward angle light scatter versus 90° light scatter by means of a bit map. More than 3,000 cells were analysed from each sample. The percentage of cells showing fluorescence greater than the background level was recorded on a one parameter histogram, measuring

fluorescence on a logarithmic scale. The mean fluorescence channel values for the positive cells were acquired from a one parameter histogram, measuring fluorescence on a linear scale. The results were analysed by the immunoanalysis program (Coulter).

2.8 Antibody purification on Synsorb beads

Synthetic beads with blood group antigens activity provide a mean for separating antibodies which have an affinity for the given immobilized oligosaccharide. Synsorb beads (Chembiomed Ltd., Edmonton, Alberta, Canada) are composed of synthetic carbohydrate structures of known specific composition covalently linked to an inert, insoluble matrix. For antibody purification, 20 ml of hybridoma culture supernatant containing antibodies were mixed with 1 g of the appropriate Synsorb beads overnight at 4°C. Unbound antibody was removed and the beads washed twice with 0.01 M PBS (pH 7.2) by centrifugation at 50 g for 5 min. The antibody was eluted by mixing the beads end over end for 15 min at room temperature with 5 ml of 2% ammonia in saline (pH 11). The beads were pelleted by centrifugation at 50 g for 5 min and the supernatant was collected and dialysed against 0.01 M PBS. The beads were stored for further use in 70% (v/v) ethanol at 4°C.

2.9 Coupling proteins to Sepharose 4B

Ulex europaeus (UEAI) lectin (Sigma, Pool, Dorset, UK) and mouse monoclonal anti-Le^a Lm112/161 provided by Dr. R.H. Fraser (Glasgow and West of Scotland

Blood Transfusion services, Law Hospital, Carluke, Lanarkshire, UK) (Fraser *et al.*, 1984) were used for the purification of molecules containing H and Le^a determinants from saliva. The lectin and the antibody (purified on Synsorb Le^a, see section 2.8) were coupled to cyanogen bromide activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden)(0.5 g of dry beads can bind up to 3.5 mg of protein). Impurities were washed away from the beads by mixing the beads with 10 ml of 1 mM HCl (pH 3) for 15 min at room temperature. The beads were pelleted by centrifugation at 50 g for 5 min. UEAI or antibody were diluted in coupling buffer (0.1 M sodium bicarbonate + 0.5 M sodium chloride, pH 9). The gel-protein mixture was rotated overnight at 4°C. Unbound material was removed by centrifugation at 50 g for 5 min. The beads were washed three times with 15 ml NET buffer (0.15 M sodium chloride + 0.04 M EDTA + 0.04 M Tris + 0.2 mM phenylmethyl sulfonyl fluoride, pH 7). Free active groups on the gel were blocked by mixing with 1 M ethanolamine (pH 8) for 120 min. Non-covalently bound material was removed by 3 cycles of washing with 15 ml 0.1 M acetate buffer (0.1 M sodium acetate + 1 M sodium chloride, pH 4) and then with 15 ml 0.1 M bicarbonate buffer (0.1 M sodium hydrogen carbonate + 1 M sodium chloride, pH 8). The gel was then used for affinity purification of blood group containing molecules from saliva.

Chapter 3

Determination of H antigen on cells and in saliva

3.1 Introduction

The H blood group antigen found on various epithelial cells has been suggested to act as an attachment site for some microorganisms (Blackwell, 1989b). Increased susceptibility of non-secretors is explained if H determinants present in secretions of secretors can bind to adhesins recognizing H; this might reduce bacterial attachment to cells from these individuals. As a first step in the investigation of this hypothesis sensitive methods for measuring the two main types of H antigen, found on buccal epithelial cells (BEC) and in saliva, have been developed.

H Type 1 and H Type 2 differ in their chemical composition, the genes that control their expression and their sites of production (for a recent review, see Clausen and Hakomori, 1989). H Type 1 is the main type of H antigen found in secretions. It is produced by fucosylation of Type 1 precursor chains. The fucosyltransferase that synthesizes H Type 1 is coded for by the *secretor* gene (Oriol *et al.*, 1981). This antigen is not found in secretions of non-secretors since they lack the *secretor* gene. The expression of H Type 2 antigen as part of the cell membrane in different tissues is independent of secretor status. The *H* gene codes for a fucosyltransferase that preferentially uses Type 2 precursor chains (Oriol *et al.*, 1981). Previous studies have shown that H Type 1 and H Type 2 antigens can be detected on the surface of BEC and in saliva (Dabelsteen *et al.*, 1982; Florry 1966; Le Pendu *et al.*, 1982; Vedtofte *et al.*, 1981).

The hypothesis that suggests H in body fluids as a protective mechanism in secretors makes two assumptions: 1) secretors and non-secretors express the same amount of H antigen on their cells and 2) secretors express large amounts of H in their secretions compared with non-secretors. To assess the validity of these two premises, the amount of H determinants on BEC, oropharyngeal cells

(OPC) and nasopharyngeal cells (NPC) was measured by flow cytometry. By this method we were able 1) to compare the expression of the two types of H determinants in secretors and non-secretors; 2) to compare the expression of these determinants on BEC, OPC and on NPC and 3) to investigate the uptake of H from saliva of secretors onto BEC. The amount of H determinant in saliva of secretors and non-secretors was determined by a simple ELISA.

3.2 Materials and methods

3.2.1 Detection of H antigen on cells

Detection of total H present (H Type 1 and 2)

Total amount of H Type 1 and H Type 2 on the surface of epithelial cells was detected by fluorescein isothiocyanate (FITC)-conjugated *Ulex europaeus* lectin (UEAI, Sigma, Poole, Dorset, UK).

Detection of H Type 2

H Type 2 was detected with mouse monoclonal antibody Lm92/90 (Mackie *et al.*, 1984). The antibody was purified on Synsorb H Type 2 (see section 2.8) and dialysed against saline for 24 hr at 4°C. The antibody was mixed with 0.4 ml of 0.5 M carbonate-bicarbonate buffer (pH 9.5) and 1ml of fluorescein isothiocyanate isomer I (Sigma) in PBS (0.4 mg/ml) was added. The mixture was rotated for 2 hr at room temperature and than dialysed for 72 hr against three changes of PBS (Johnson and Holborow, 1986).

Test procedure

Two hundred microlitres of cell suspension containing $2.5x10^5$ cells/ml were mixed with an equal volume of FITC labelled UEAI, monoclonal antibody or buffer in a 5 ml culture tube and incubated at 37°C for 15–60 min with continuous shaking. The cells were washed three times with DPBS+B (300 g for 10 min) and fixed with 0.2 ml of 1% buffered paraformaldehyde (Lanier and Warner, 1981). The cells were analysed on an EPICS'C' flow cytometer as described in section 2.7.

3.2.2 Adsorption of H from saliva

Two hundred microlitres of a suspension of BEC ($2x10^5$ cells/ml) from secretors or non-secretors were mixed with 200–600 μl of undiluted secretor saliva from a blood group O donor, who did not express Lewis antigen, in 5 ml test tubes. The tubes were shaken for 30–120 min at 37°C and washed twice with DPBS+B (300 g for 10 min). The amount of H antigen on the cells was determined using UEAI and monoclonal antibody as described above.

3.2.3 Detection of H antigen in saliva

The composition of buffers used in this assay are given in section 2.6. The wells of polystyrene microtiter plates (M129B Dynatech, Billinghurst, Sussex, UK) were coated overnight at 4°C, with 100 μl of ten fold dilutions of saliva in coating buffer. All further procedures were carried out at room temperature. The wells were washed three times with washing buffer then blocked for 15

min with blocking buffer. The blocking buffer was removed and the wells were washed twice with washing buffer. One hundred microlitres of biotinylated UEAI (Sigma) diluted in blocking buffer (5 μg/ml) were added to each well for 30 min. After 3 washes, 100 μl of a 1/10 or 1/100 dilution of streptavidin biotinylated horseradish peroxidase complex (Amersham, Amersham, UK) in blocking buffer were added for 20 min. The wells were washed 3 times, and 100 μl of substrate solution were added. The reaction was allowed to develop in the dark for 20 min and then stopped by adding 50 μl of 12.5% (v/v) H_2SO_4. The absorbance at 490 nm was measured using a Dynatech plate reader. Samples were run in duplicate and the readings averaged.

3.2.4 Statistics

The Student's t-test was used for all statistical analysis.

3.3 Results

3.3.1 Detection of H antigen on cells

Ulex concentration

Ten fold dilutions of *Ulex* lectin were incubated with BEC from a secretor and from a non-secretor for 30 min. The levels of H determinant detected are shown in Figure 3.1. Above 10 μg/ml, all the cells in the two populations were positive; but those from the secretor had higher levels of H (mean fluorescence). Below

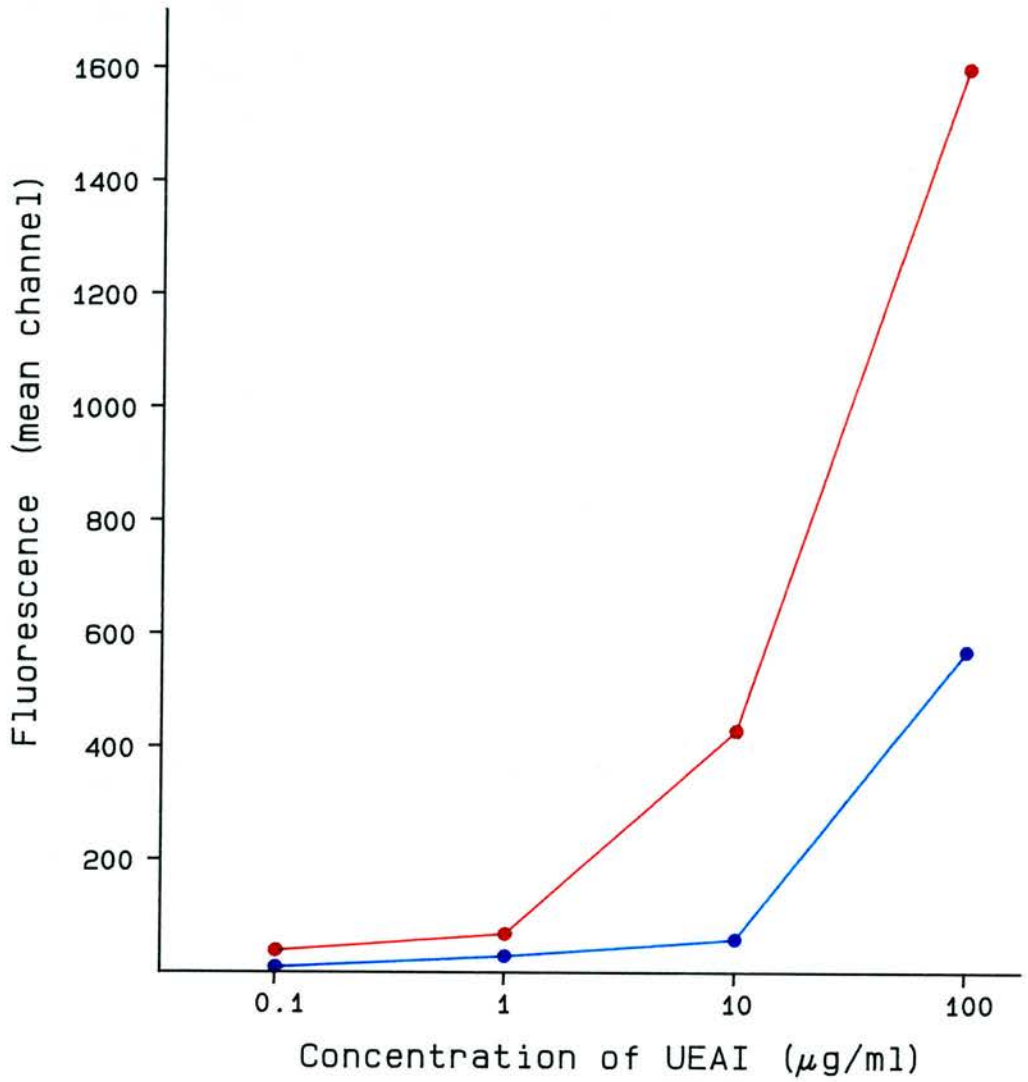

Figure 3.1: Determination of H on BEC from secretor (red) and non-secretor (blue) using UEAI and an incubation period of 30 min. Each point represents the results from a secretor and a non-secretor donor.

10 μg/ml there was a steady drop in the percentage of positive cells. UEAI concentrations were used in subsequent assays at 10 μg/ml or more.

Time course

Variation in the amount of fluorescence with incubation period is shown in Figure 3.2. At the concentration of *Ulex* used (30 μg/ml) an incubation period greater than 15 min did not result in an increase in the amount of H detected on cells from the secretor. This point also showed the biggest difference between the secretor and the non-secretor cells. In the subsequent experiments, cells were incubated with UEAI for 15 min.

Total amount of H on cells from secretors and non-secretors

Figure 3.3 shows the levels of H antigen detected with UEAI on BEC from secretors and non-secretors measured by flow cytometry. The total amount of H antigen on BEC was between 3.5–6 times greater for secretors than for non-secretors, depending on UEAI concentration. This difference was statistically significant for the three concentrations of UEAI used in this study ($p<0.001$). Almost all the BEC (90–99%) in the two populations expressed the H antigen; but, a greater amount of antigen, as determined by mean channel, was present on secretor cells. Figure 3.4 shows two distinct peaks for the two populations of BEC analysed. Cells obtained from a non-secretor (peak 'a') show a lower mean fluorescence compared to cells from a secretor donor (peak 'b'). More than 90% of the cells in each population show fluorescence levels higher than the background level, indicated by the cursor on the left of the histogram. A similar pattern of expression of H determinants was observed for oropharyngeal

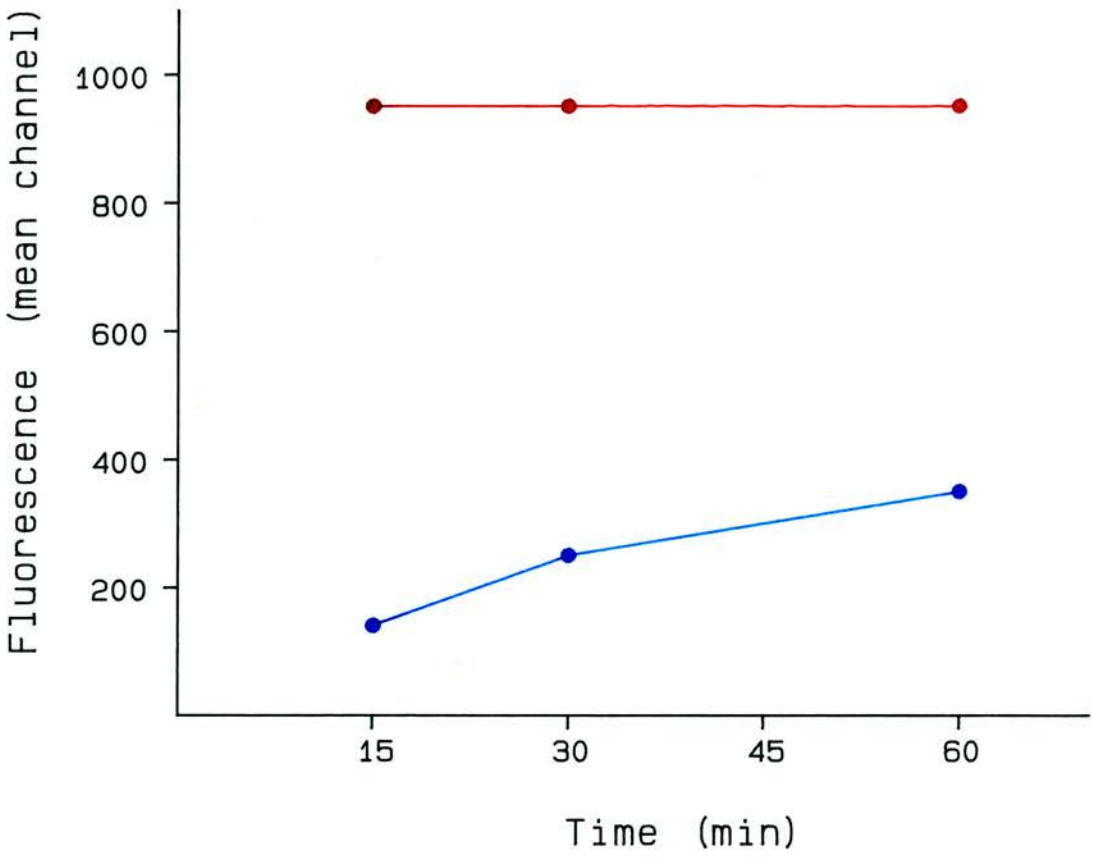

Figure 3.2: Time course for determination of H on BEC from secretor (red) and non-secretor (blue) using UEAI. Each point represents the results from a secretor and a non-secretor donor.

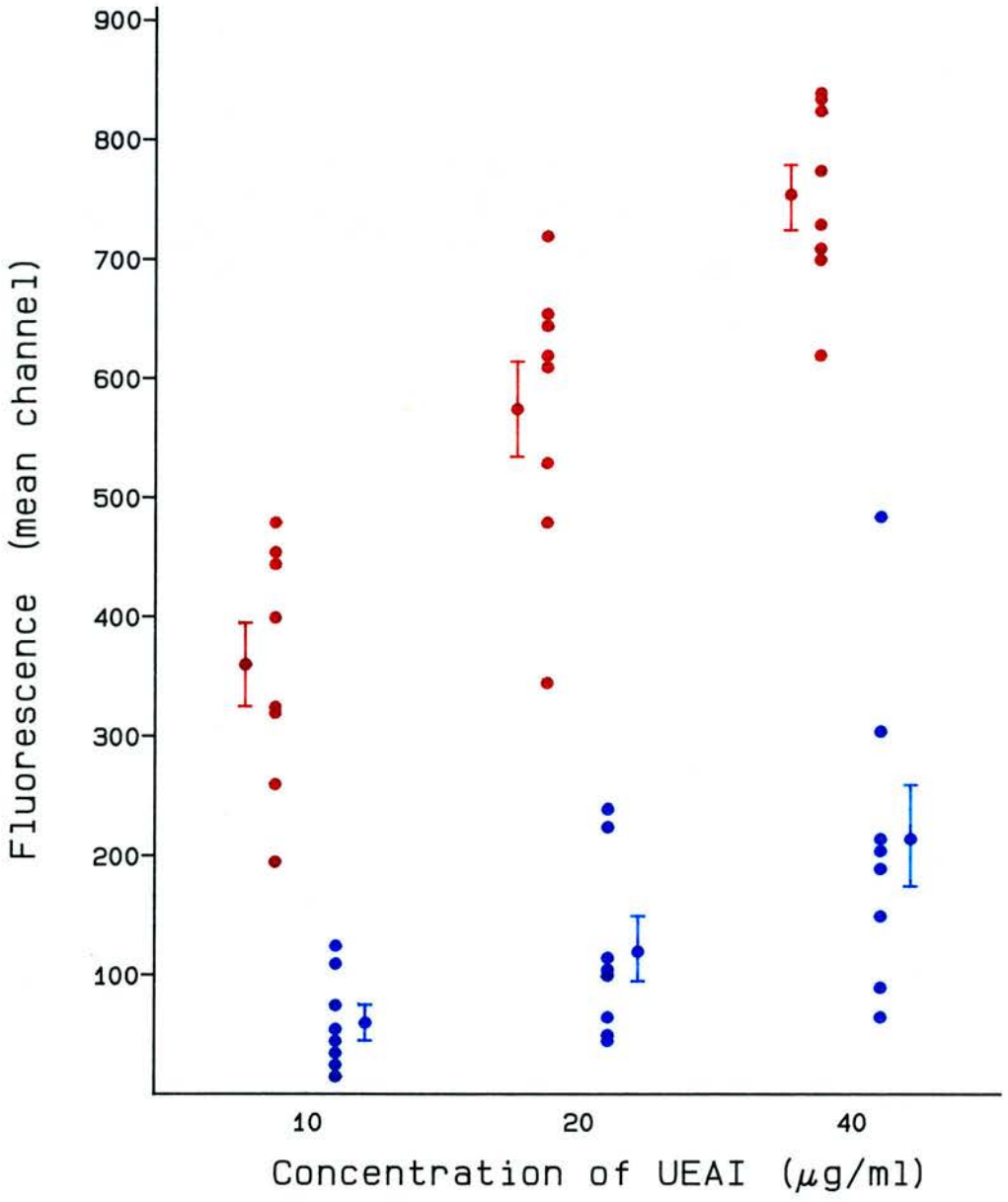

Figure 3.3: Flow cytometric determination of H on BEC from secretors (red) and non-secretors (blue). Points represents the mean fluorescence of cells obtained from different individuals. Bars represent ± 1SEM of 7–8 individuals tested.

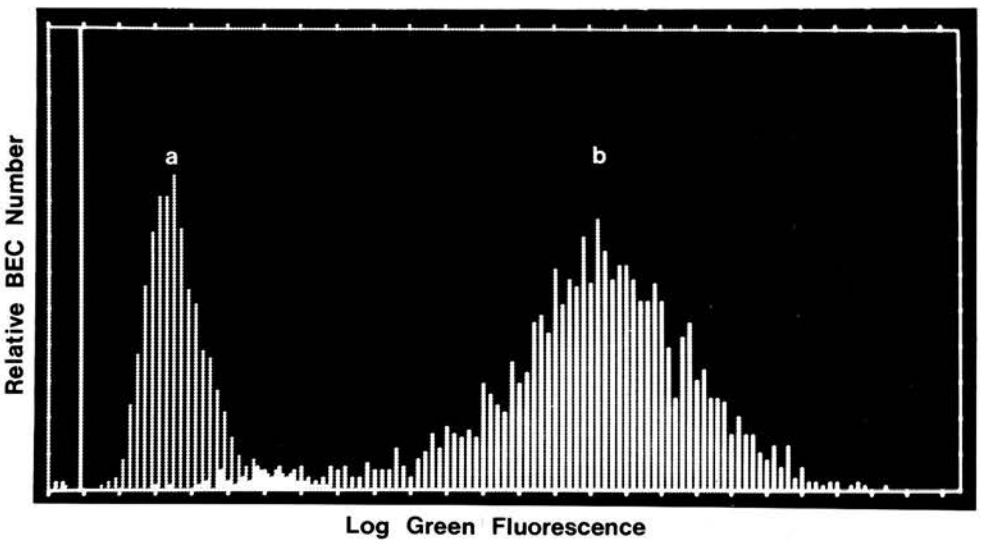

Figure 3.4: Flow cytometric comparision of UEAI (20 μg/ml) binding to non-secretor (a) and secretor (b) BEC. Cursor set to give 1% background level.

cells (OPC) and nasopharyngeal cells (NPC) from a secretor and a non-secretor (Figure 3.5). For all three types of epithelial cells tested, cells from secretors expressed higher levels of H compared with cells from a non-secretor.

Detection of H Type 2 on BEC

There was no significant difference in the amount of H Type 2 on BEC of secretors and non-secretors, as detected by monoclonal antibody used at two concentrations (Figure 3.6).

3.3.2 Adsorption of H onto BEC

Incubation period and saliva volume

Detection of H on BEC from a non-secretor after incubation with saliva specimens from secretors are shown in Figure 3.7. Under the experimental conditions, the amount of saliva used and the length of the incubation did not influence significantly the amount of H detected on the cells. In the following experiments 200 μl of saliva were incubated with cells for 1 hr.

H levels after incubation with secretors' saliva

The levels of total H and H Type 2 on cells from secretors and non-secretors before and after incubation with saliva from a secretor are shown in Figure 3.8. After incubation of BEC from non-secretors with secretor saliva, there was a statistically significant increase ($p<0.005$) in the amount of H antigen detected

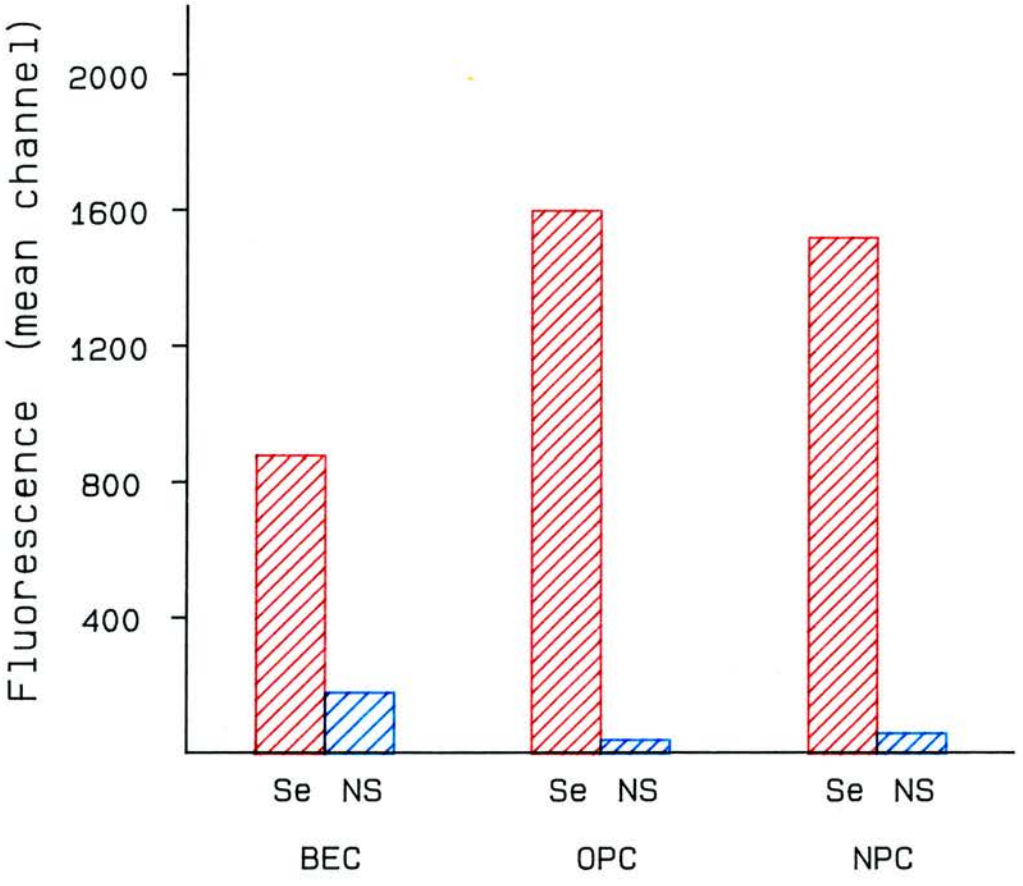

Figure 3.5: Detection of H using UEAI (20 μg/ml) on buccal epithelial cells (BEC), oropharyngeal cells (OPC) and nasopharyngeal cells (NPC) from a secretor (Se) and a non-secretor (NS).

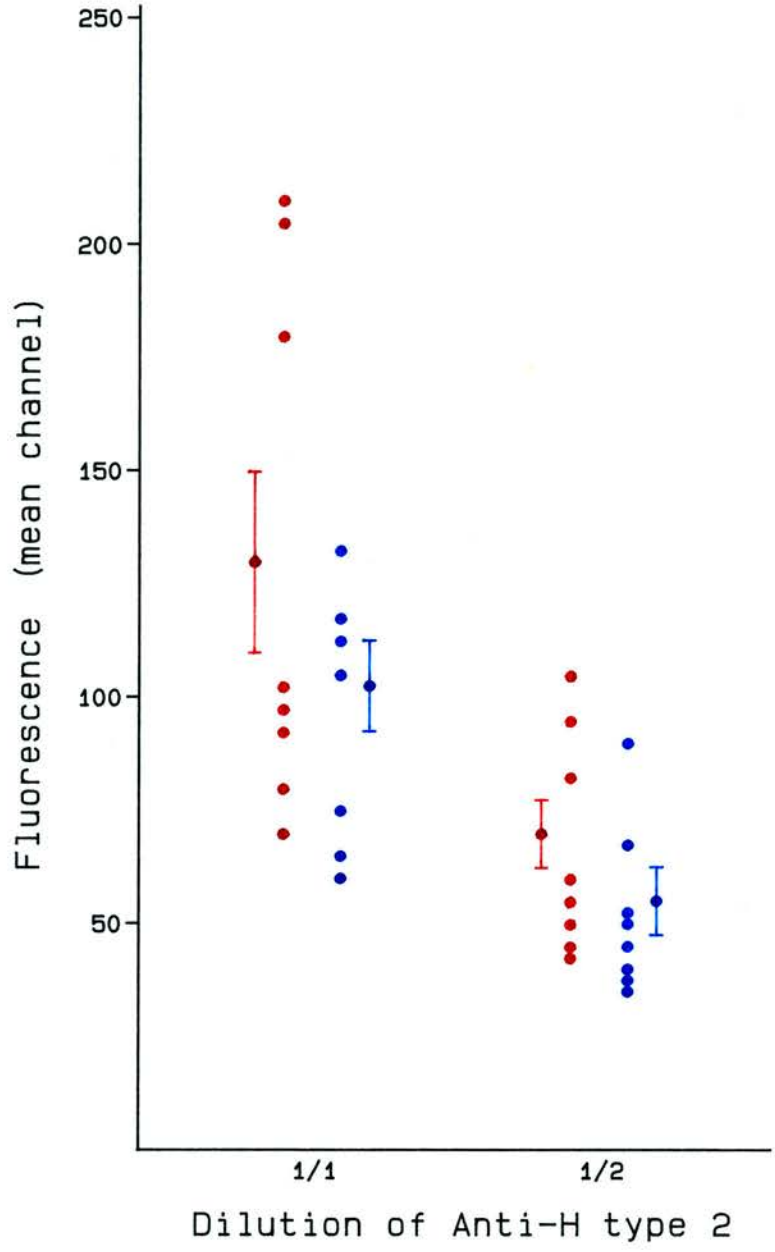

Figure 3.6: Determination of H Type 2 on BEC from secretors (red) and non-secretors (blue). Points represents the mean fluorescence of cells obtained from different individuals. Bars represent ±1SEM of 7-8 individuals tested.

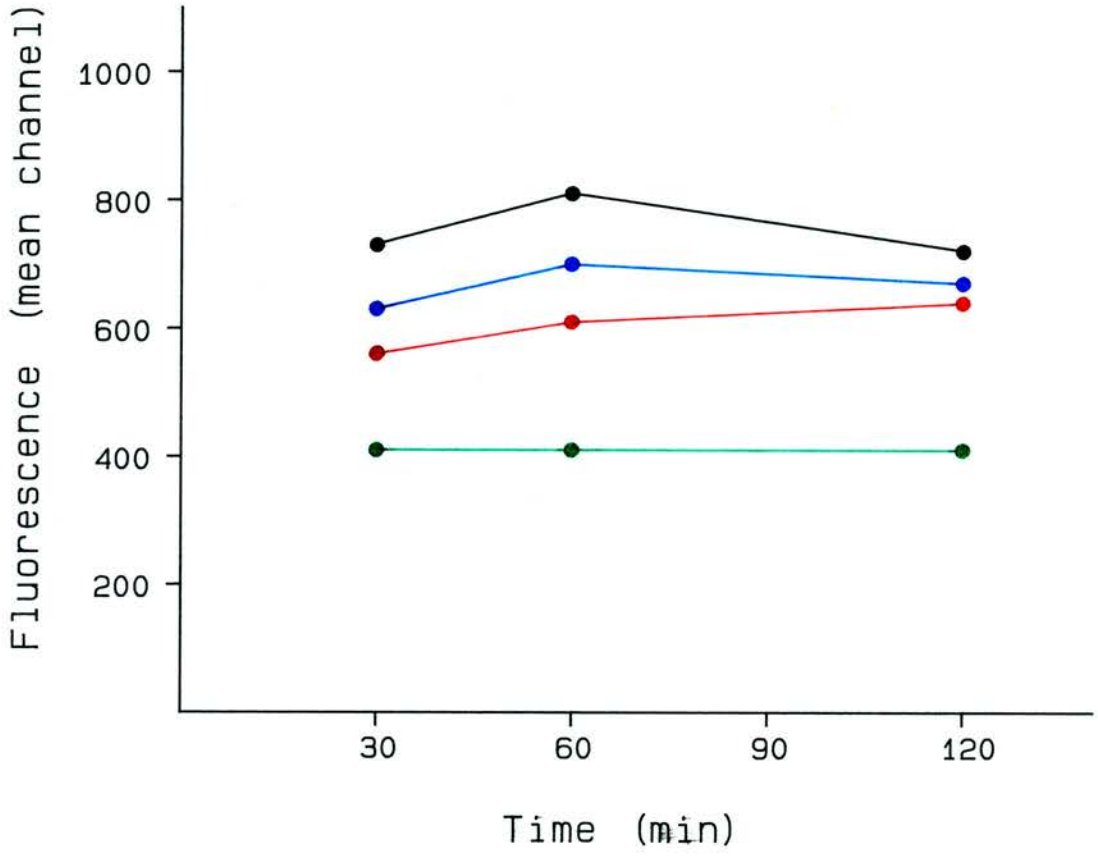

Figure 3.7: Detection of H by UEAI on BEC from a non-secretor after incubation with buffer (green), 200 μl (black), 400 μl (blue) and 600 μl (red) of secretors' saliva.

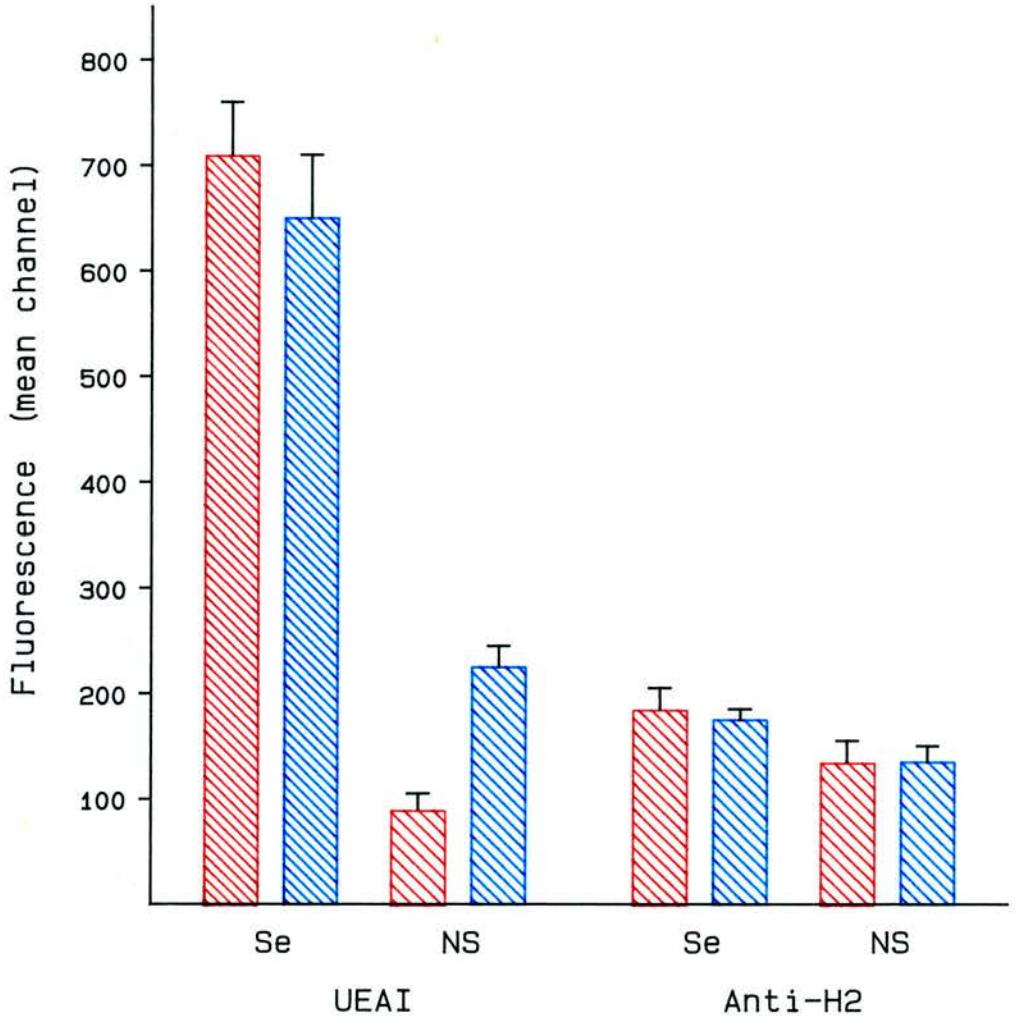

Figure 3.8: Determination of H by UEAI and anti-H Type 2 on secretor's and non-secretor's BEC before (red) and after (blue) incubation with secretors' saliva. Bars represent ±1SEM of four experiments.

by UEAI. There was no significant change in the levels of H when secretor cells were incubated with secretor saliva. The amount of H Type 2 antigen on the cells of either secretors or non-secretors, did not change significantly following incubation with saliva.

3.3.3 Detection of H in saliva

Streptavidin dilution

The amount of streptavidin required to detect the maximum level of H was determined by varying the streptavidin concentration at two dilutions of saliva (Figure 3.9). The maximum amount of H in the two dilutions of secretor's saliva was obtained at a dilution of 1/100 of streptavidin. In the following experiments, streptavidin was used at 1/100 dilution.

H levels in saliva of secretors and non-secretors

The presence of H determinant can be measured in secretors' saliva at a dilution of 10^5 by the ELISA system developed (Figure 3.10). At all dilutions of saliva used, saliva from non-secretors contained significantly lower levels of H ($p<0.001$).

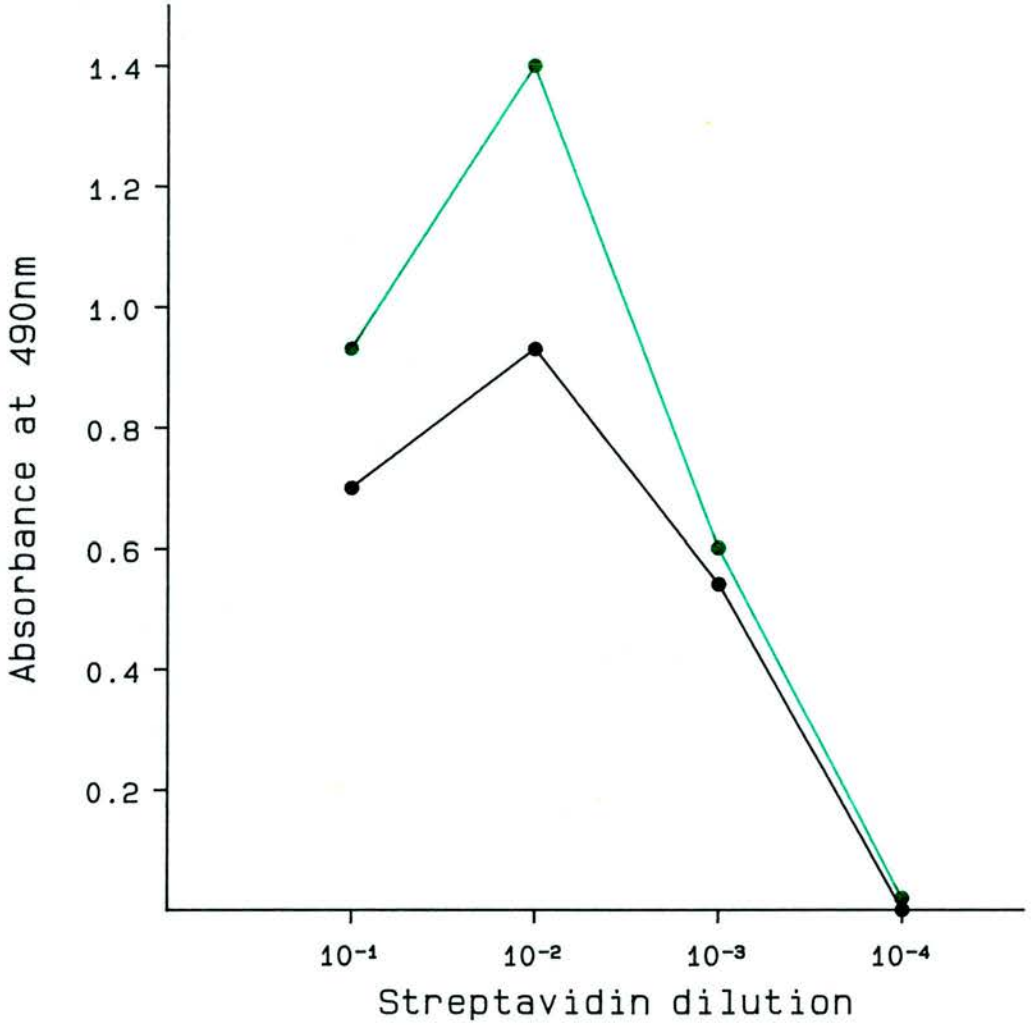

Figure 3.9: Detection of H by ELISA in secretor's saliva diluted 1/10 (black) and 1/100 (green) at various dilutions of streptavidin. Each point represents the result from a secretor donor.

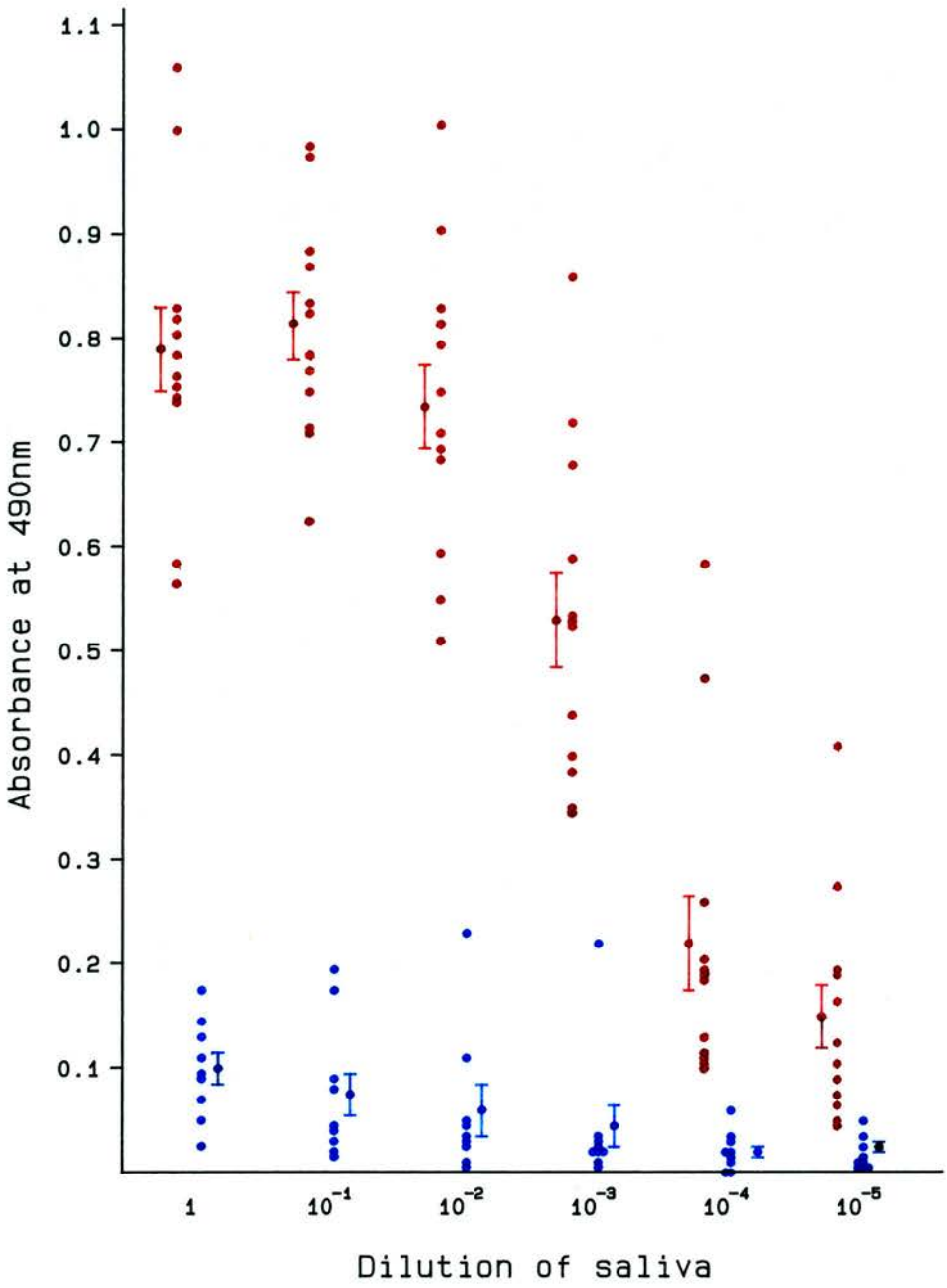

Figure 3.10: Measurement of H in saliva from secretors (red) and non-secretors (blue) by ELISA. Points represents the mean fluorescence of cells obtained from different individuals. Bars represent ±1SEM of 9–12 individuals tested.

3.4 Discussion

Two sensitive methods for the determination of H antigen were developed in this study. Development of these methods was the initial step in the investigation of the expression of H determinants on cells and in secretions of secretors and non-secretors. This information was needed to examine the hypothesis that H might be a receptor for bacteria. Flow cytometric analysis of BEC with FITC-labelled UEAI measured the total amount of H antigen; and with monoclonal antibody to H Type 2, the amount of H Type 2 antigen was determined. The biotin streptavidin ELISA can be used to measure the amount of H in saliva. UEAI detects both H Type 2 and H Type 1; it can agglutinate red blood cells by their H Type 2, and this agglutination can be inhibited by H Type 1 in secretor saliva. This lectin does not react with the native H Type 1 but with deacetylated H Type 1 (Hindsgaul *et al.*, 1985); therefore, the definition of secretor status is based on deacetylated H Type 1 and not the native structure. In this study H Type 1 refers to the deacetylated form since *Ulex europaeus* was used for the detection of H.

In flow cytometry analyses, virtually all BEC from secretors and non-secretors express the H determinant; however, as indicated by the mean fluorescence level measured, secretors and non-secretors differ in the amount of antigen expressed on their cells. Depending on the concentration of UEAI used, secretors had between 3.5–6 times more H determinant on their BEC surface than non-secretors. Similarly, NPC and OPC from a secretor had higher levels of H than cells from similar sites obtained from a non-secretor. In contrast, there was no difference in the amount of H Type 2 detected on cells of secretors compared with non-secretors using the monoclonal antibody. The difference in the level of H on BEC from the two groups appears to be due to H Type 1. These observations

support the work of Oriol *et al.* (1981). According to their hypothesis, there are two fucosyltransferases responsible for the production of H antigen; one is controlled by the *secretor* gene, preferentially reacting with Type 1 chains; and the other, controlled by the *H* gene, preferentially reacts with Type 2 chains. Since both secretors and non-secretors express the *H* gene product, no difference was found in the amount of H Type 2 on cells of secretors compared with non-secretors.

In this study the increased amount of H on secretors cells is attributed to H Type 1 resulting from the action of the *secretor* gene product. Incubation of non-secretor cells with secretor saliva showed that molecules containing the H determinant can be adsorbed from saliva onto the cell surface. The increase in the amount of H was detected with UEAI and not with anti-H Type 2. These results suggest that H Type 1 antigen present in secretor saliva can be adsorbed onto the epithelial cell surfaces in a way similar to that described for the Lewis antigen (Marcus and Cass, 1969). This explains the difference found in the amount of H detected by *Ulex* on cells of secretors and non-secretors. Although, as suggested by Le Pendu *et al.* (1982), the *secretor* gene product might be able to fucosylate precursor Type 2 chain, the results presented here indicate that the H Type 2 antigen is not present in saliva. The level of H Type 2 on BEC from non-secretors did not increase after incubation with secretor's saliva (Figure 3.8). BEC of secretors did not show an increase in the amount of H following incubation with saliva, suggesting their cell surfaces are already saturated with this antigen. Since saliva contains glycoproteins and small amounts of glycolipids carrying blood group determinants, the nature of the carriers of the blood group determinants adsorbed by the cells is not known.

The ELISA developed in this study provides a simple and sensitive method

to measure the amount of H present in saliva and to determine secretor status. In contrast to the haemagglutination inhibition test, commonly used for determination of secretor state, this method is quantitative rather than qualitative. With the ELISA system, a large number of samples can be analysed in a very short time without the requirement for fresh human red blood cells. At a 1/10 dilution of saliva, large differences in the amount of H in specimens from secretors and non-secretors were clearly demonstrable. The small amount of H detected in non-secretors' saliva is probably due to H antigen derived from epithelium or contaminating blood cells.

One of the hypotheses investigated in this study proposes that H in secretions of secretors can reduce colonization of epithelial surfaces of secretors. The results described in this chapter confirm the premise that secretors have high levels of H determinant in their secretions compared with non-secretors. The results contradict the second premise that the amounts of H on cells of secretors and non-secretors are similar; cells from secretors were found to express more H than cells from non-secretors. If H is an attachment site for the bacteria, cells of secretors should bind more bacteria than cells of non-secretors. H can be proposed as a receptor to explain the decreased proportion of secretors among patients, if H in secretions has higher affinity for the bacteria than H on cells or if H is found in very high levels in secretions. To further investigate the role of H as a receptor for bacterial binding, the attachment of bacteria to BEC from secretors and non-secretors was investigated.

There are additional applications for this method to studies in oncology. The blood group antigens normally present in epithelium are reported to be partially or completely lost by neoplastic cells (Dabelsteen and Pindborg, 1973; Davidsohn *et al.*, 1966; Prendergast *et al.*, 1968). Accurate determination of blood

group antigens expressed on cells by the method described in this study might be useful in early diagnosis of malignancies as previously suggested (Dabelsteen and Fulling, 1971; Davidsohn *et al.*, 1969; Davidsohn *et al.*, 1971).

This ELISA for detection of H can also be applied to the determination of secretor status using other body fluids such as nasopharyngeal secretions, in which there might be problems of low concentrations of antigen and small volumes of specimens.

Chapter 4

Attachment of meningococci to epithelial cells

4.1 Introduction

Specific adherence of microorganisms to epithelial cells is an important step in the colonization process. Although non-specific factors such as surface charge, pH, ionic bridging and hydrophobic interactions might be important (Watt and Ward, 1980), it has been argued that the attachment of bacteria to host cells is facilitated by specific interactions. Specific interaction might be necessary since the surfaces of both host and bacteria have a net negative charge. This mutual repulsion could be overcome by interaction of surface molecules which act as ligands (Ofek and Beachey, 1980). The observation that some microorganisms show site or cell specificity, i.e. selectively binding to a receptor on certain cell types, supports this hypothesis. This has been demonstrated by Stephens and McGee (1981) for *N. meningitidis*, which bind specifically to human nasopharyngeal cells.

Early investigators of meningococcal pathogenesis implicated the nasopharynx as the natural habitat of the meningococcus. This was suggested to be the site from which the organism was transmitted to other individuals and as the initial site of mucosal multiplication (see Stephens and Whitney, 1985). Once infection is established, internalization of the bacteria by epithelial cells and transport of the organisms across the normally protective mucosal barrier is possible (Stephens, 1989). Colonization can result in asymptomatic meningococcal carriage; in non-epidemic periods, about 5–10% of the population carry the bacteria in the nasopharynx without adverse effects (Greenfield *et al.*, 1971).

Despite the importance of colonization in the pathogenesis of meningococcal disease, only recently has this aspect of the biology of *N. meningitidis* been studied. The host factors involved in the attachment of meningococci are still

poorly understood. An epidemiological study of an outbreak of meningitis in a school found that non-secretors of blood group antigens were over-represented among carriers of meningococci (Blackwell *et al.*, 1990). This data suggests that differences at the mucosal surfaces of secretors and non-secretors might contribute to differences in colonization by the bacteria and, consequently, to disease susceptibility.

A flow cytometric assay was developed to examine the hypothesis that there might be differences between secretors and non-secretors in the number or distribution of ligands involved in bacterial attachment to epithelial cells. Using this assay the attachment of bacteria to epithelial cells from the two populations was compared.

4.2 Materials and methods

4.2.1 Detection of bound bacteria with antibody

Two strains of *N. meningitidis* were used to standardize the binding assay: serogroup C serotype 2b serosubtype P1.2 (C:2b:P1.2) and serogroup B serotype 15 serosubtype P1.16 (B:15:P1.16). Bacteria grown overnight were collected from MNYC plates, suspended in PBS and washed three times by centrifugation at 1000 g for 20 min. The bacterial concentration was adjusted to give a BEC:bacteria ratio of 1:5000 in the final mixture. Two hundred microlitres of BEC were mixed with 100 μl of the bacterial suspension or buffer in 5 ml culture tubes and incubated at 37°C for 30 min with continuous shaking. All further incubations were carried out at 4°C with continuous shaking. The cells were

washed three times with DPBS+B and 200 μl of the same buffer containing 5% normal rabbit serum was added for 15 min. After washing once with DPBS+B (300 g for 10 min), 100 μl of rabbit polyclonal meningococcal serogrouping serum (serogroup C and serogroup B) (Wellcome, Beckenham, UK) diluted in DPBS+B were added and incubated for 30 min. As a control, buffer was added in place of the antibody. Cells were washed three times with DPBS+B (300 g for 10 min) and 100 μl of FITC conjugated donkey anti-rabbit immunoglobulin (Scottish Antibody Production Unit, Carluke, Lanarkshire) in DPBS+B added to each tube. The mixtures were incubated for 30 min and washed three times with DPBS+B. The cells were fixed with 1% buffered paraformaldehyde and analysed in an EPICS'C' flow cytometer (see section 2.7). For each sample analysed two parameters were obtained; 1) the percentage of cells in the population that showed fluorescence levels higher than the background (cells incubated without bacteria); 2) the mean level of fluorescence in the population of positive cells, reflecting the mean number of bacteria bound to the cells.

4.2.2 Labelling the bacteria with FITC

4.2.2.1 Method 1

The first technique is a modification of the method described for labelling antibody with FITC (Johnson and Holborow, 1986). A heavy suspension of bacteria in PBS was prepared from colonies grown overnight on MNYC plates. Bacteria were washed three times in PBS by centrifugation at 1000 g for 20 min. Four hundred micrograms of FITC were dissolved in 1 ml of PBS and sonicated in an ultrasonic-bath (Astell Scientific, London, UK) for 5 min. Two hundred microlitres of 1 M carbonate-bicarbonate buffer (pH 8.9) were mixed with 100 μl

of the FITC mixture, added to the pellet of bacteria and incubated at 37°C for 2 hr with continuous shaking. Free FITC was removed by washing three times with PBS at 1000 g for 20 min.

4.2.2.2 Method 2

This method is a modification of that described by Wright and Jong (1986). A heavy suspension of bacteria in PBS was prepared and washed as described above. Four hundred micrograms of FITC were dissolved in 1 ml buffer (0.05 M sodium carbonate + 0.1 M sodium chloride, pH 9.2) and added to the bacteria pellet. The mixture was incubated at 37°C for 20 min and washed three times as described for method 1.

4.2.3 Attachment assay

N. meningitidis serogroup C strains directly conjugated with FITC were used in this assay. After labelling, the bacterial concentration was adjusted to produce the required ratios of epithelial cells to bacteria in the final mixture. Two hundred microlitres of epithelial cells were mixed with 100 μl of each dilution of bacteria or buffer in a 5 ml culture tube and incubated at 37°C for 0–120 min with continuous shaking. The cells were washed three times with DPBS+B by centrifugation at 300 g for 10 min and fixed with 1% (v/v) buffered paraformaldehyde. Experiments were performed to study the effect on meningococcal attachment of: time, pH, fixation of BEC and sonication of BEC. The bacteria were mixed with the BEC for periods ranging from 0 to 120 min. The effect of pH on attachment was assessed by adjustment of the DPBS+B buffer to the desired pH by the addition of NaOH or HCl. Fixation was examined by treatment of

the BEC with buffered paraformaldehyde for 20 min prior to incubation with bacteria. The BEC were washed twice in DPBS+B before adding the bacteria. The effect of sonicating BEC before incubation with bacteria was studied by sonicating the cells in an ultrasonic-bath (Astell Scientific, London, UK) for 5 min prior to adding the bacteria. The role of Ca^{2+} ions was determined by washing the BEC twice with DPBS containing 10 mM EDTA before adding the bacteria in DPBS+EDTA (10 mM).

The binding of different serotypes of *N. meningitidis* to BEC and attachment to cells from secretors and non-secretors were also studied.

The samples were analysed by the flow cytometer as described in section 2.7. Results were expressed as the mean fluorescence level or by an attachment index obtained by multiplication of the percentage of positive cells by the mean fluorescence. This value gives an indication of the total fluorescence in a cell sample.

4.2.4 Statistics

For comparison of attachment of meningococci to BEC of secretors and non-secretors, the results for the non-secretor were expressed as percentage of the secretor, calculated by the formula: [(non-secretor - secretor) / secretor] x 100. These values were used in the Student-t test to calculate confidence intervals.

4.3 Results

4.3.1 Detection of bacterial binding with antibodies

Bound bacteria were detected with a rabbit anti-capsular antiserum. The presence of bound antibody was determined with FITC conjugated antibody to rabbit immunoglobulin. Three controls, without bacteria, were included: cells alone, cells with FITC conjugated anti-rabbit immunoglobulin and cells with both antibodies. The fluorescence levels of control cells incubated with antibodies and test cells incubated with bacteria and antibodies were compared to the background fluorescence level (cells only). As shown in Table 4.1, the fluorescence levels obtained for the controls with the antibodies were higher than the background level. More than 70% of the cells in the two samples bound the FITC conjugated antibody. A higher percentage of positive cells was found after incubation with anti-capsule and anti-rabbit antibodies compared with cells incubated with anti-rabbit antibody only. The values obtained for cells that had been incubated with bacteria were not markedly different from the controls for the two strains of bacteria used. These results indicated that both antibodies used were binding to structures on the BEC. Since higher readings were found for the controls this method could not be used for the study of bacterial attachment to BEC.

4.3.2 Labelling bacteria with FITC

Two methods for directly labelling the bacteria with FITC were compared. It can be seen from Figure 4.1 that the mean fluorescence level of attachment when

Table 4.1: Detection of bacterial binding to BEC using antibodies

Cells	Group C		Group B	
	%	mean	%	mean
Cells A:				
Control 1	77	150	77	150
Control 2	95	230	87	185
Test	73	261	90	190
Cells B:				
Control 1	72	218	72	218
Control 2	81	150	78	156
Test	71	124	64	122

BEC from two individuals (cells A and cells B) were incubated with two strains of bacteria B:15:P1.16 (group B) and C:2b:P1.2 (group C). Two controls were used: BEC + anti-rabbit immunoglobulin (control 1) and BEC + anti-bacteria + anti-rabbit immunoglobulin (control 2). In the test a BEC:bacteria ratio of 1:5000 was used.

bacteria were labelled by method 2 was higher than with method 1 (3.5 times). The attachment of bacteria to BEC was more uniform with the second staining method. The second method is also more convenient to use since the sonication stage is not required and the incubation period is shorter. In the following experiments the second method was used to label the bacteria with fluorescein isothiocyanate.

4.3.3 Bacterial binding

4.3.3.1 Time course

The attachment of bacteria to BEC from a secretor donor was measured using 80 and 40 bacteria per cell at four incubation periods (Figure 4.2). A similar pattern was obtained for the two ratios of bacteria per cell used. No attachment was observed when the BEC and bacteria were mixed and immediately washed (time 0). Attachment increases rapidly over the first 60 min after which the rate appears to slow down. Incubation for 30 min gave significant attachment to BEC at the two ratios used. This point is also in the middle of the initial rapid binding period and will allow detection of increases or decreases in attachment. Therefore, 30 min was used as the incubation period in the following experiments.

4.3.3.2 The effect of pH

The attachment of bacteria to BEC, at the ratio of 40 bacteria per cell, did not show a significant pH optimum over the range tested (Figure 4.3), therefore in

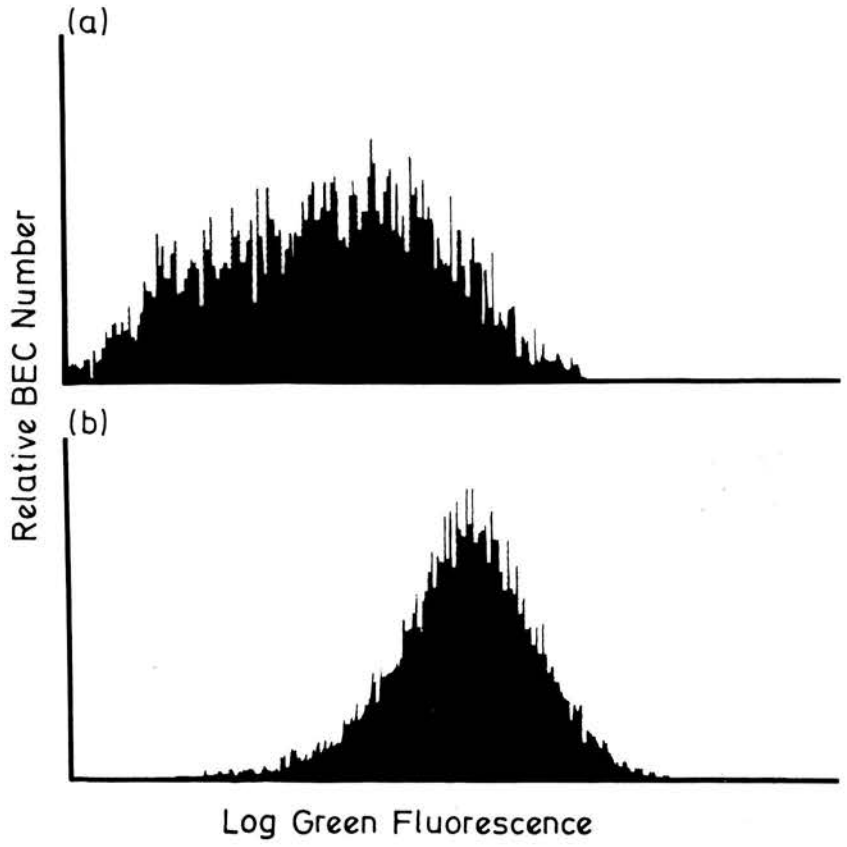

Figure 4.1: Flow cytometric comparision of bacterial binding to BEC using FITC conjugated bacteria prepared by: method 1 (a) and method 2 (b). The peaks represent cells with fluorescence level higher then the background (no bacteria).

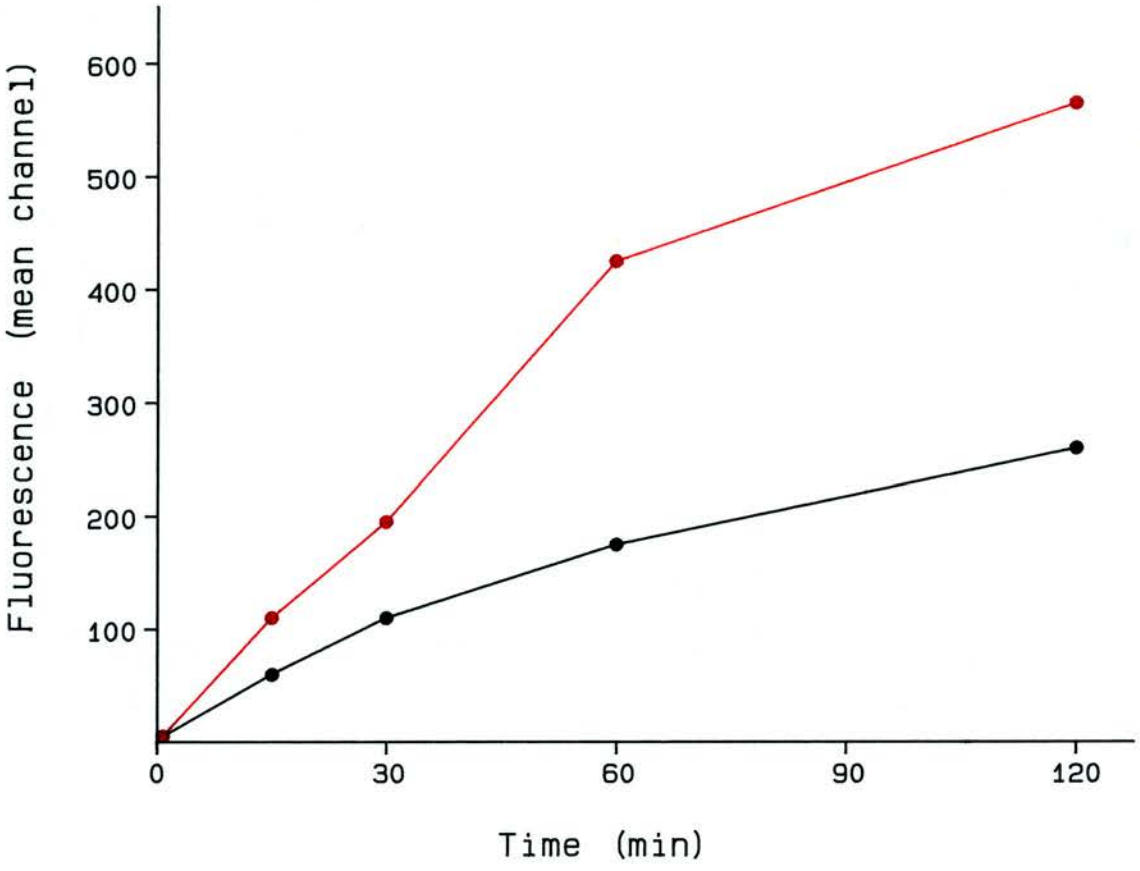

Figure 4.2: The effect of time on the attachment of FITC labelled *N. meningitidis* (C:2b:P1.2) to BEC. The level of attachment was determined by flow cytometry after the BEC were incubated with 40 (black) or 80 (red) bacteria per cell.

the following experiments the physiological pH (7.2) was used.

4.3.4 Attachment of meningococci to BEC and pharyngeal cells

Binding of *N. meningitidis* to BEC, and cells obtained from the oropharynx and the nasopharynx was demonstrated (Table 4.2). The three populations of cells differ in their size, therefore the attachment level (mean fluorescence) is not comparable. Since BEC are easier to obtain, they were further used in this study.

4.3.5 Attachment of three strains of meningococci to BEC

Three serotypes of *N. meningitidis* serogroup C (2b, 4 and 2b.P1.2) were tested for their ability to bind to BEC by the attachment assay developed in this study. The three strains used showed a similar pattern of bacterial attachment at the three doses of bacteria used and under the following experimental conditions: incubation at 37°C for 30 min, pH 7.2 and in the presence of Ca^{2+} and Mg^{2+} ions (Figure 4.4).

4.3.6 Attachment of meningococci to BEC from secretors and non-secretors

The attachment of *N. meningitidis* serogroup C to BEC from secretors and non-secretors was compared at three ratios of bacteria per cell. For each BEC sample

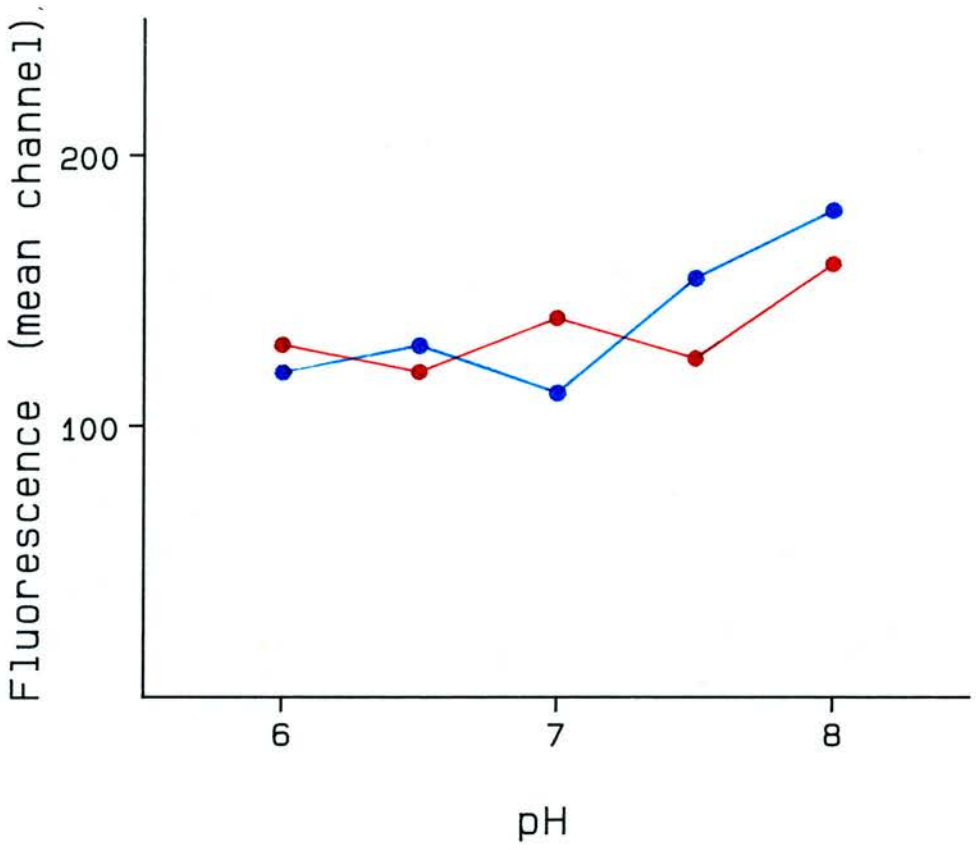

Figure 4.3: The effect of pH on the attachment of FITC labelled *N. meningitidis* (C:2b:P1.2) to BEC from a secretor (red) and a non-secretor (blue), detected by flow cytometer.

Table 4.2: Attachment of bacteria to BEC, OPC, and NPC

	BEC	OPC	NPC
%	99	84	94
Mean	670	115	262

The percentage of positive cells and the mean level of fluorescence obtained after incubation of epithelial cells with bacteria at a ratio of 80 bacteria per cell. The epithelial cells used: buccal epithelial cells (BEC), oropharyngeal cells (OPC) and nasopharyngeal cells (NPC), were obtained from a non-secretor donor.

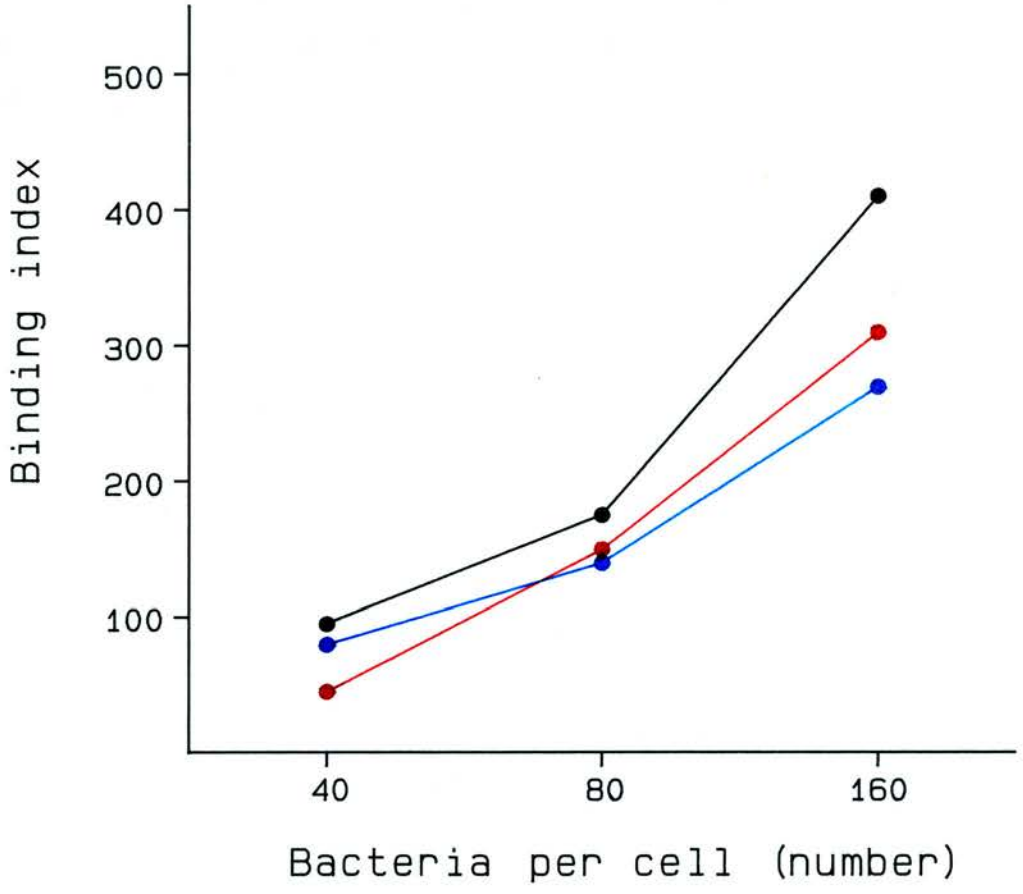

Figure 4.4: Binding of three strains of *N. meningitidis*: C:2a (black), C:4 (red) and C:2b:P1.2 (blue), to BEC from one donor. Similar results obtained for BEC from three other donors.

an index was obtained as described in section 4.2.3. The indices were used in Figure 4.5 to compare bacterial attachment between pairs of secretors and non-secretors. The attachment to non-secretor cells was expressed as a percentage of the secretor cells. Although at the lower ratios of bacteria to cell, BEC of non-secretors bound more bacteria than those of secretors, the difference was not statistically significant at any of the three ratios used. As the number of bacteria per cell decreased, the mean of the difference increased, indicating more bacteria were attached to BEC from non-secretors than to BEC from secretors. The 95% confidence limit, represented by the bars, indicates the probability that non-secretors cells bind more bacteria than secretors cells. Reducing the bacteria to cell ratio resulted in an increased probability that non-secretors cells bind more bacteria. At the lowest ratio used, the results were just outside the 95% confidence level.

4.3.7 The effect of fixation, EDTA and sonication on bacterial binding

Treating the cells with buffered paraformaldehyde, sonication or EDTA reduced the attachment of bacteria to cells (Table 4.3). Fixation of cells prior to incubation with bacteria reduced the mean level of bacterial binding per cell by up to 60% compared with the attachment to untreated cells. The effect of treating the cells by sonication or EDTA was not as strong as that observed for fixation (between 40–50% decrease). The same pattern was observed for treatment of cells obtained from secretor and non-secretor donors.

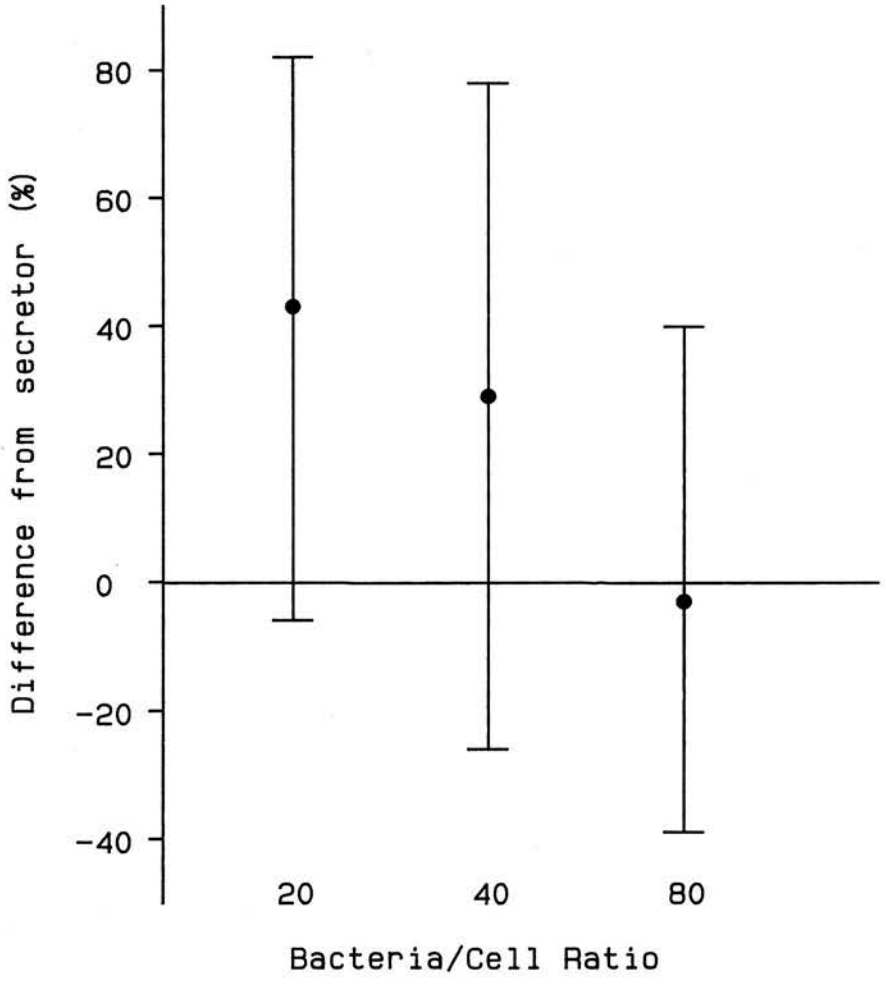

Figure 4.5: Attachment of *N. meningitidis* C:2b:P1.2 to BEC from secretors and non-secretors. Each point represents the average difference between 8 pairs of secretors and non-secretors. The bars show 95% confidence limit.

Table 4.3: The effect of fixation, sonication and EDTA on attachment to BEC

Cells	Mean fluorescence			
	Normal	Fixed	Sonicated	EDTA
Secretor	180	67	125	109
Non-secretor	193	88	93.2	120

Mean fluorescence levels obtained for BEC from a secretor and a non-secretor donor, when cells were not treated (normal), fixed with buffered paraformaldehyde, sonicated or treated with EDTA.

4.4 Discussion

The early stage of infection with pathogenic Neisseria is determined by receptor-mediated events that result in colonization and invasion of human mucosal tissues. Despite the importance of this initial step in carriage of the bacteria and in infection, very little is known about the molecules interacting in the process of attachment. A major problem in studying the pathogenesis of meningococci is the specificity of the disease for humans. This fact has limited the use of animal models in studies of the pathogenesis of this organism. The potential severity of the diseases caused by meningococci has limited direct human experimentation. To overcome this problem, the attachment of meningococci to epithelial cells has been studied *in vitro* by cell and organ culture assays involving predominantly cells and tissue of human origin.

Human buccal epithelial cells have been favoured for use in attachment assays because they are easily obtained. However, there are several factors to be considered: 1) the cells obtained by scraping the buccal mucosa are not uniform in the degree of maturation, viability and size; 2) the structure of the cells is influenced by hormonal factors and other variables that are not easily controlled; 3) many of the cells already have bacteria (oral flora) attached to their surfaces and 4) the buccal mucosa is not normally colonized by *Neisseria meningitidis*. Despite those drawbacks, BEC have been extensively used in studies of meningococcal attachment (Trust *et al.*, 1983; Salit and Morton, 1981; Stephens and Whitney, 1985; Stephens *et al.*, 1988).

In addition, the attachment of *N. meningitidis* to erythrocytes (Trust *et al.*, 1983; Salit and Morton, 1981), nasopharyngeal cells (Stephens *et al.*, 1983), oropharyngeal cells (Salit and Morton, 1981) and nasopharyngeal tissue in organ

culture (Stephens *et al.*, 1987) has been studied.

In this study we compared the attachment of meningococci to secretors and non-secretors of ABO blood group antigens, which required examination of a large number of donors from both populations. In this case, the availability of cells is an important factor. The aim of this study was to test the influence of blood group determinants associated with the expression of the *Se* gene (Le^a and H) on the attachment of *N. meningitidis* to epithelial cells. Therefore, in section 3.2.1, the expression of H determinant on pharyngeal cells (the natural site for colonization by meningococci) and BEC was compared. As was shown in Figure 3.5, BEC and pharyngeal cells of a secretor expressed higher levels of H determinants compared with the cells of a non-secretor. The level of expression of ABH blood group antigens on both types of cells seems to be under the control of the *Se* gene. Although BEC are not the natural site for colonization by meningococci, they appear to be suitable model for studying the influence of the expression of these determinants on attachment.

As a first step in setting up the attachment assay, an indirect method for detection of bound bacteria with antibodies was tested. As shown in Table 4.1, this system gave high background due to cross-reactivity between bacterial antibodies and antigens present on the BEC. Therefore, another system for detection of bound bacteria was developed in which bacteria were directly conjugated with FITC.

Bacterial attachment was observed using buccal epithelial, nasopharyngeal and oropharyngeal cells from a non-secretor (Table 4.2), but the levels were not comparable since the cells differ in their size. These results are in agreement with previous studies that have demonstrated attachment of *N. meningitidis* to these three types of cells (Salit and Morton, 1981).

In previous studies, quantification of attachment of meningococci to human cells was tested by several methods. Haemagglutination was used for determination of attachment to erythrocytes. This test was also used to detect the expression of pili on the bacterial surface (Trust *et al.*, 1983). Radiolabelling the bacteria was used to quantitate the attachment of meningococci to BEC. This method was used to determine the proportion of bacteria that bind to the cells, but did not give information for the proportion and distribution of attachment on the cells (Trust *et al.*, 1983). A common method used for the assessment of bacterial binding to cells is the use of light microscopy to detect bound bacteria after staining by Gram's method (Salit and Morton, 1981). With this method relatively small number of cells are examined (50–100) and there are problems differentiating between the normal flora and meningococci (Salit and Morton, 1981).

In this study a flow cytometric method was developed for determination of attachment of fluorescein-labelled bacteria to epithelial cells. Using this method the percentage of cells that bind bacteria as well as the mean level of binding are obtained. This method overcame some of the drawbacks associated with the use of BEC for attachment studies. First, unlike counting the number of bacteria bound to cells by light microscopy, attachment assessed by the flow cytometer is objective. Second, large number of cells (>3,000 cells in each sample) can be analysed in a short period of time (100 cells in a second). This allows rapid examination of large numbers of cells from a large group of donors. In addition, the method overcame the wide variation in the numbers of bacteria bound to cells obtained from an individual. As indicated by the standard error, the variation is very low (less then 3% of the mean fluorescence). Variation between donors resulting from hormonal changes and other factors are still a problem which is difficult to control; but a large population of donors should compen-

sate for these variations. To reduce as much as possible variation resulting from differences between individuals, secretors and non-secretors tested were paired according to their ABO blood group, sex and age. The sample size was limited by the number of non-secretor donors available. Hence, difficulties in obtaining significant differences in attachment to BEC between the two populations might result from the wide variation in the population.

Under the experimental conditions used, attachment of bacteria to BEC was decreased when the cells were fixed, sonicated or treated with EDTA (Table 4.3). Fixation of cells influences the fluidity of the cell membrane. This could effect the ability of the receptors to move in the membrane and therefore interfere with bacterial binding. The presence of Ca^{2+} and Mg^{2+} ions seems to play a role in part of the interactions between the bacteria and the cells. This might indicate the involvment of lectin-like interactions, in which the presence of Ca^{2+} is known to be required (Cook and Bugg, 1975). Sonication of the cells before attachment reduced bacterial binding. This could result from elimination of some of the receptors on the cells surface in the process of sonication. After setting up the experimental conditions, attachment of one strain of *N. meningitidis* to BEC from secretors was compared with attachment to BEC from non-secretors. The serogroup C strain used is one isolated recently from a patient in Scotland where the presence of disease due to group C strains is increasing (R.J. Fallon, personal communication).

Two hypotheses were tested in this study. The first suggested that bacteria have adhesins that can bind to the H epitopes. In the second, the Le^a determinant is proposed as the receptor for bacterial binding. If the first is correct, cells from secretors should bind more bacteria than cells from non-secretors, since secretors express far more H determinants on their cells compared to non-secretors

(see section 3.3.1). If Le^a is the receptor, since non-secretors express far more Le^a on their cells compared with secretors (Vedtofte, 1985), they should bind more bacteria. In the attachment experiments, BEC from non-secretors were found to bind more bacteria than cells from secretors; the difference was not statistically significant ($0.05<p<0.1$), but may support the hypothesis that Le^a is a receptor for the bacteria.

Although the Le^a determinant is found in greater quantity among non-secretors, it is present in both secretors and non-secretors. These results might reflect the variable amounts of Le^a present on cells of secretors. Although secretors express Le^b predominantly, some of these individuals have substantial amounts of Le^a in their body fluids (Ogata *et al.*, 1988) and, therefore, on their epithelial cells.

As the ratio of bacteria to epithelial cells was decreased, the increased binding of meningococci to cells of non-secretors became more apparent. In our system, incubation of BEC with bacteria at a ratio of 1:20 is the lowest ratio that gives fluorescence levels above the background. These lower ratios might reflect more closely the *in vivo* situation in which the numbers of bacteria per cell are limited.

Higher levels of attachment to epithelial cells from non-secretors compared with cells from secretors was reported for uropathogenic strains of *E. coli* (Lomberg *et al.*, 1986) and *Candida* blastospores (May *et al.*, 1986). Urinary tract infections due to *E. coli* and superficial infections due to *Candida* have been associated with non-secretion of blood group antigens (Blackwell *et al.*, 1989b; May *et al.*, 1989; Thom *et al.*, 1989).

Most of the experiments in this chapter used one strain of meningococci for developing the assay and for testing the attachment to secretors and non-

secretors cells. The results shown in Figure 4.4 indicate that the system is suitable for testing other strains; and the results obtained for the one strain may be extended for other strains and species of bacteria with the assay developed.

Since higher levels of attachment were obtained with cells from non-secretors, we concluded that H is not a binding site for the strain tested.

Chapter 5

Possible role for Lewis^a determinant as a binding site for meningococci

5.1 Introduction

In chapter 4, BEC from non-secretors were found to bind more bacteria than BEC from secretors. The only determinant known to be present in higher levels on cells from non-secretors compared with cells from secretors is the Le^a blood group determinant. Therefore the role of this structure as a receptor for bacterial binding was further investigated.

The Lewis determinants are found as glycosphingolipids and glycoproteins in serum and body secretions. This antigen is not produced endogenously by red blood cells; it is adsorbed from serum onto the cells (Marcus and Cass, 1969). The antigens have been shown to be present on many tissues including buccal epithelium (Vedtofte, 1985).

The type of Lewis determinant expressed in an individual depends on the presence of the *Se* gene. The Lewis determinants are produced by fucosylation of precursor Type 1 or H Type 1 by a fucosyltransferase coded by the *Le* gene. This gene is expressed in 90–93% of the population and is inherited independently of the *Se* gene. The type of acceptor molecule used will determine the type of Lewis determinant produced. Fucosylation of precursor Type 1 results in the expression of Le^a and when H Type 1 is fucosylated Le^b is produced. The *Se* and *Le* gene products can both fucosylate precursor Type 1 (see Figure 1.5). When the two genes are expressed (i.e. in secretors, Lewis-positive individuals), the ratio of expression of the two types of Le determinants depends on the efficiency of the two enzymes. In most cases, the Se enzyme is more efficient; therefore, most of the precursor molecules are converted to H Type 1 and then to Le^b. When the Se enzyme is less efficient, a larger proportion of the precursor chain is converted to Le^a which cannot be further fucosylated by the Se enzyme. Hence,

in secretors varying amounts of Le^a and Le^b are present; but in non-secretors, since the *Se* gene is not expressed, only Le^a is produced.

In this part of the study, a role for the Le^a determinant in bacterial attachment was investigated by two methods. 1) The ability of bacteria to adsorb partially purified Le^a from suspension was examined. If the bacteria have adhesins that can bind Le^a, incubation of bacteria with molecules containing this determinant should result in a decrease in the amount of Le^a determinants in the preparations. To measure the amount of Le^a in solution an ELISA method was developed. 2) Pretreatment of the bacteria with partially purified molecules containing Le^a prior to incubation with BEC was studied. This was compared to pretreatment of bacteria with saliva.

5.2 Materials and methods

5.2.1 Development of an ELISA to measure Le^a

Composition of the buffers used in the ELISA are given in section 2.6.

5.2.1.1 Coating the plate with anti-mouse immunoglobulin antibody

The wells of polystyrene microtitre plates (M129B Dynatech, Billinghurst, Sussex, UK) were coated overnight at 4°C with 100 μl of rabbit anti-mouse immunoglobulin (DAKO Ltd., Bucks, UK) in coating buffer (60 μg/ml). All further procedures were carried out at room temperature. The wells were washed three times with washing buffer and incubated with 150 μl of blocking buffer for 15

min. The buffer was then removed and the wells were washed twice with washing buffer. Mouse monoclonal anti-Le^a Lm112/161 (100 μl) provided by Dr. R.H. Fraser (Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, Lanarkshire, UK) (Fraser *et al.*, 1984) in blocking buffer (100 μl) was added for 1 hr. Unbound antibody was removed by washing three times with washing buffer. One hundred microlitres of ten fold dilutions of saliva in blocking buffer were added for 30 min. After washing three times with washing buffer, 100 μl of a goat anti-Le^a serum (Behring, Marburg, W. Germany) diluted 1/100 in blocking buffer were added for 30 min. The wells were washed 3 times and 100 μl of horseradish peroxidase-conjugated donkey anti-goat immunoglobulin (Scottish Antibody Production Unit, Carluke, Lanarkshire, UK) diluted 1/250 in blocking buffer added. The plate was incubated for 2 hr, washed 3 times and 100 μl of substrate solution added. The reaction was allowed to develop in the dark for 20 min and stopped by adding 50 μl of 12.5% (v/v) H_2SO_4. Absorbance at 490 nm was measured with a Dynatech plate reader. Samples were tested in duplicate and the readings averaged.

5.2.1.2 Coating the plate with Synsorb purified antibody

The method described above was modified so that the plate was coated overnight at 4°C with monoclonal anti-Le^a that had been purified on Synsorb Le^a (see section 2.8). After washing and blocking as described above, 100 μl of test samples diluted in blocking buffer were added for 30 min. Unbound antigen was removed by washing 3 times with washing buffer. The polyclonal anti-Le^a was added and the rest of the procedures were carried out as described above.

5.2.2 Purification of Le^a containing molecules

A modification of the method described by Pak *et al.* (1984) was used to isolate Le^a containing molecules from saliva of a non-secretor donor. Unstimulated saliva specimens (20 ml) were collected from healthy individuals and centrifuged at 1000 g for 20 min. The supernatant was collected, boiled for 20 min, recentrifuged, freeze dried and resuspended in 6 ml of distilled water. The concentrated non-secretor saliva (6 ml) was mixed with 1.5 ml of swollen anti-Le^a Sepharose 4B beads(see section 2.9). After overnight incubation at 4°C the supernatant was removed and the Sepharose beads washed three times with 15 ml NET buffer (0.15 M NaCl, 0.04 M ethylenediamine tetraacetic acid, 0.04 M Tris, 0.2 mM phenylmethylsulfonyl fluoride, pH 7). The antigens were eluted by incubation with 3 ml of 1 M acetic acid for 30 min at 20°C with continuous rotation. The eluate was dialysed against 3 changes of PBS. The presence of Le^a determinants was tested using the ELISA system as described in section 5.2.1.2.

5.2.3 Adsorption of Le^a by bacteria

The strains of bacteria used in this assay are shown in Table 5.1. Preparations of molecules containing Le^a determinants (150 μl) were incubated with an equal volume of bacterial suspension (10^5 bacteria per ml) or buffer in V well microtitre plates (M25A Dynatech). The positive control was antigen preparation with buffer and the negative control was bacteria with buffer. The plate was incubated for 2 hr at 37°C in 5–10% CO_2 on a plate shaker (Titertek, Flow Laboratories, Rickmansworth, Herts, UK). The bacteria were pelleted at 1000 g for 20 min, the supernatant collected and the amount of Le^a determinants present measured by ELISA (see section 5.2.1.2). Two fold dilutions (100 μl) of the

Table 5.1: Neisseria meningitidis isolates tested

Number tested	Serogroup	Serotype	Subtype
1	C	2b	P1.2
1	C	-	P1.3
2	B	15	P1.16
1	*NG	15	P1.16

*Non-groupable

supernatant were added to separate wells for 30 min (as the saliva in the ELISA method). The amount of Le^a epitope left in the supernatant was expressed as a percentage of the amount of the epitope in the positive control (antigen+PBS).

5.2.4 Inhibition of bacterial attachment to BEC

FITC-labelled bacteria prepared as described in section 4.2.2 (method 2) were adjusted to $5x10^7$ bacteria per ml. Bacterial suspensions (0.9 ml) were mixed with 0.7 ml of the following: affinity purified Le^a preparation diluted 1/3 or 1/10 in PBS; undiluted saliva from a non-secretor and saliva diluted 1/10 or 1/100 in PBS; PBS diluent as a control. After 30 min incubation at 37°C with continuous shaking, free antigen was removed by addition of 10 ml of PBS followed by centrifugation at 1000 g for 20 min. The supernatant was removed from the bacteria by suction to leave a volume of 1 ml. The pellet was resuspended in this volume. One hundred microlitres of these suspensions were mixed with 200 μl of BEC from a non-secretor or a secretor to give ratios 1:100 or 1:150 BEC to bacteria. The attachment assay was performed as described in section 4.2.3. Samples were tested in duplicate, analysed by flow cytometry (see section 2.7) and readings were averaged.

5.2.5 Statistics

In some experiments, the results were expressed as percentage of the control, calculated by the formula: [(test - control) / control] x 100. These values were used in the Student t-test to calculate p values.

5.3 Results

5.3.1 Development of an ELISA to measure Le^a

The monoclonal anti-Le^a used is supplied as a tissue culture supernatant containing foetal calf serum which can compete with the antibody for space on the plate. Therefore, in the first method anti-mouse immunoglobulin was used to capture the mouse monoclonal anti-Le^a. By this method, the readings for undiluted saliva and saliva diluted 1/100 from a non-secretor were more than double the readings for saliva from a secretor at the same dilutions (Figure 5.1). A small difference was observed when saliva was diluted 10^3 times but no difference was found with 10^4 dilution. The second method used the same mouse monoclonal anti-Le^a but after purification on Synsorb-Le^a beads as the first stage capture antibody. Compared to the previous method, this method gave greater differences in the levels of Le^a determinants measured between the secretor and non-secretor donors (Figure 5.2). This system was more sensitive detecting Le^a determinants in saliva from a non-secretor at the 10^{-4} dilution and was therefore used in subsequent experiments for detection of Le^a in solutions. Saliva from a secretor donor, whose red blood cells typed Le^{a-b-}, was used as a negative control. Low readings were observed for this saliva compared with samples from the secretor and the non-secretor donors, who expresed Lewis antigens on their erythrocytes. These readings are probably due to non-specific interactions. In Figure 5.3, Le^a levels in saliva from 12 secretor and non-secretor individuals at the 10^{-2} dilution are shown. Non-secretors have significantly ($p<0.001$) higher levels of Le^a determinants compared to secretors. At this dilution about 5 times more Le^a determinants were detected in saliva from non-secretors compared with saliva from secretors.

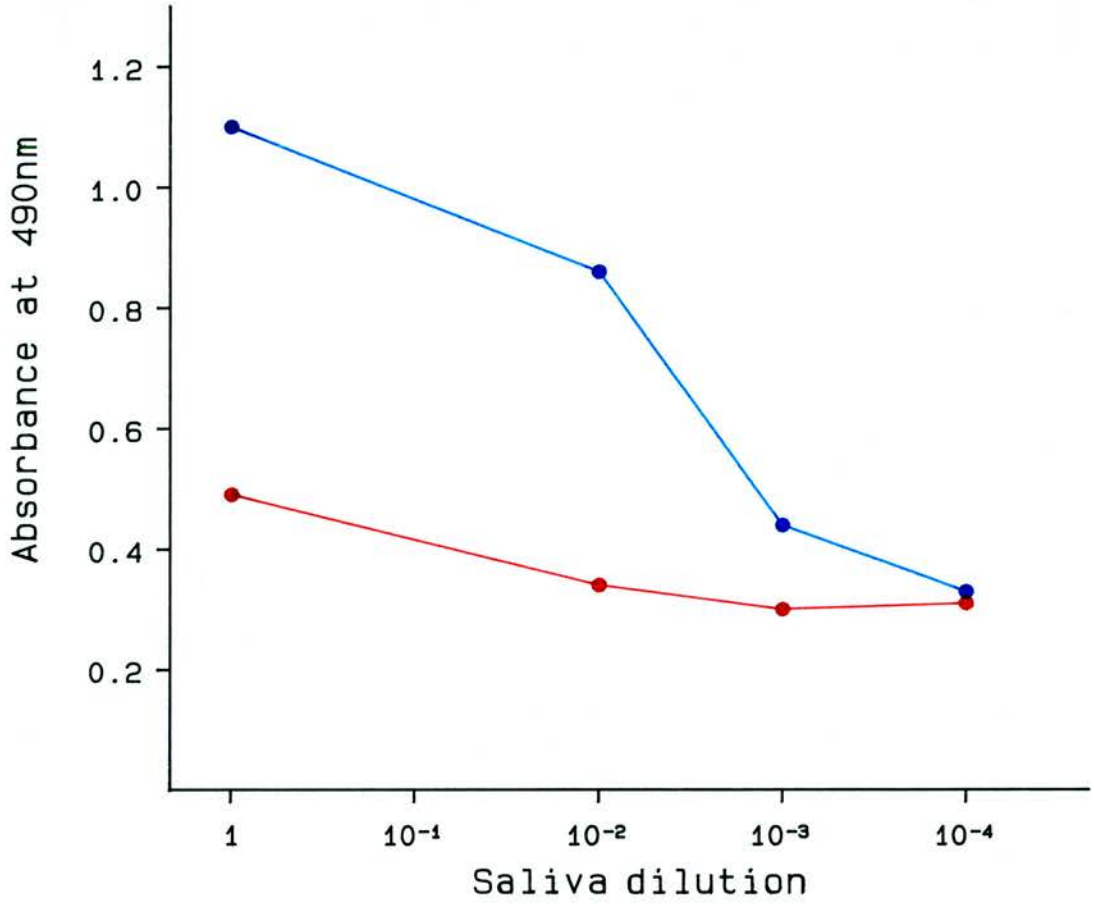

Figure 5.1: Detection of Le^a determinants in saliva from a secretor (red) and a non-secretor (blue) by ELISA. Monoclonal anti-Le^a in culture supernatant was captured by coating the wells with anti-mouse immunoglobulin antibody.

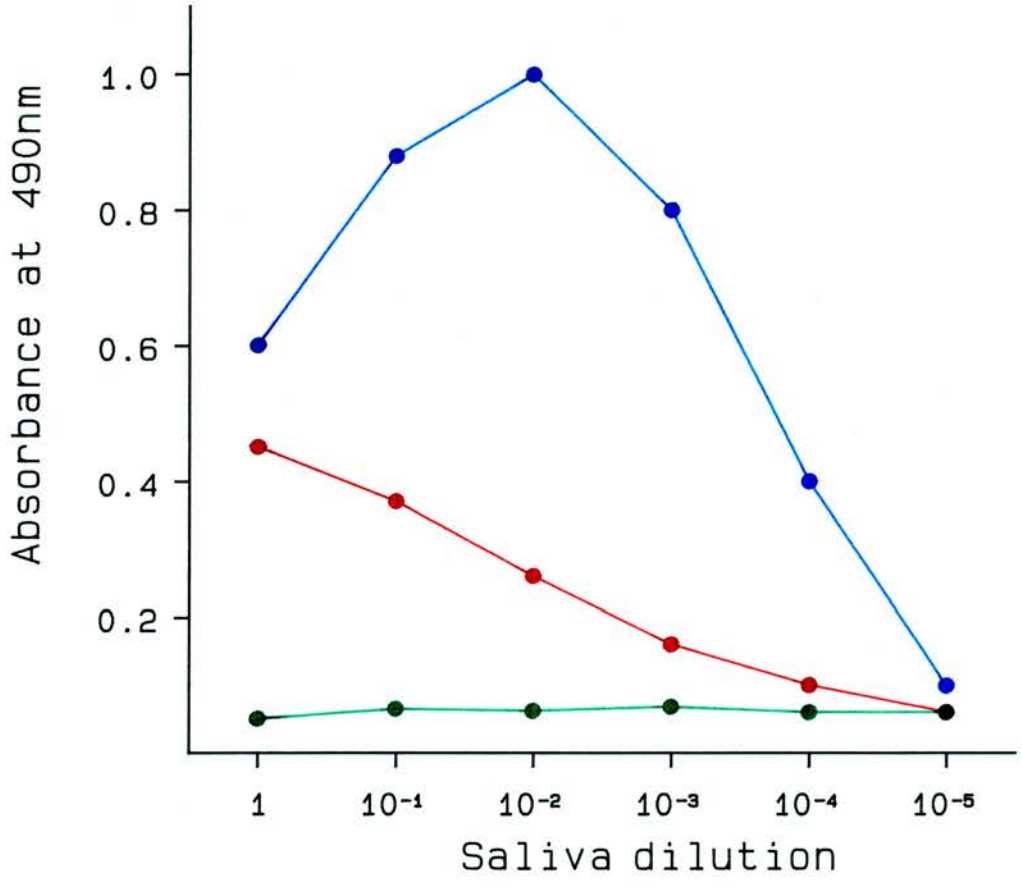

Figure 5.2: Detection of Le^a determinants by ELISA in saliva from individuals of three phenotypes: non-secretor Lewis-positive (blue); secretor Lewis-positive (red) and a secretor Lewis-negative (green). Synsorb beads were used to capture anti-Le^a from the culture supernatant and the wells were coated with this purified antibody.

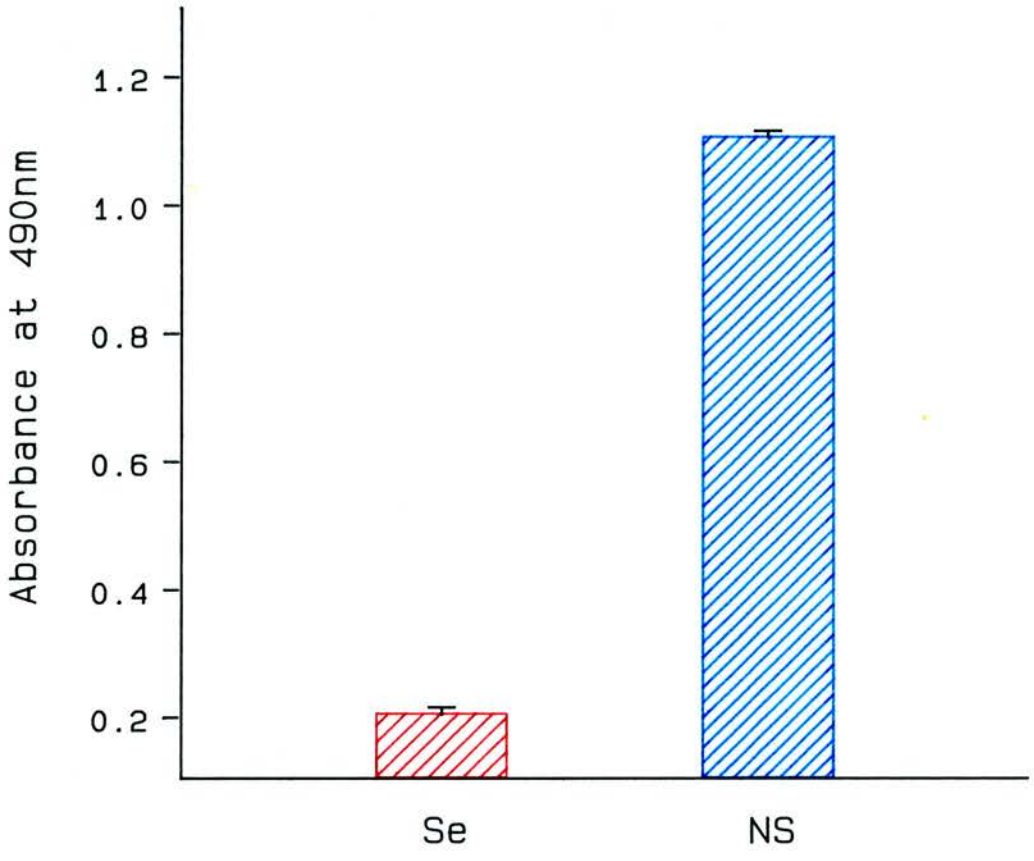

Figure 5.3: Mean levels of Le^a detected by ELISA in saliva (diluted 1/100) from 12 secretor and 12 non-secretor donors. The bars represent $\pm$1SEM.

5.3.2 Determination of Le^a in solution after purification

Saliva from a non-secretor was applied to Sepharose-Le^a beads. The eluate from these beads contained detectable levels of Le^a determinants (Figure 5.4). The level measured in the undiluted preparation was higher than in the original supernatant, probably as a result of other proteins present in the supernatant (concentrated saliva) that might interfere with the interaction of antigen and antibody in the ELISA system. The supernatant from the Sepharose still contained high levels of Le^a determinants and was used in further cycles for purification of Le^a containing molecules.

5.3.3 Adsorption of Le^a antigen by bacteria

The ability of bacteria to interact with Le^a was investigated by incubating preparations containing Le^a with bacteria and measuring the remaining amount of Le^a by the ELISA system. After incubation with bacteria, there was a decrease in the amount of Le^a containing molecules in the preparation (Figure 5.5). Statistically significant differences ($p<0.025$) were observed at the 1/4 and 1/8 dilutions. The optical density values for the control ranged between 0.3 and 0.65 depending on the antigen dilution. The results obtained with the 5 different strains tested were pooled together since there was no apparent difference in the results obtained with the different serogroups or serotypes.

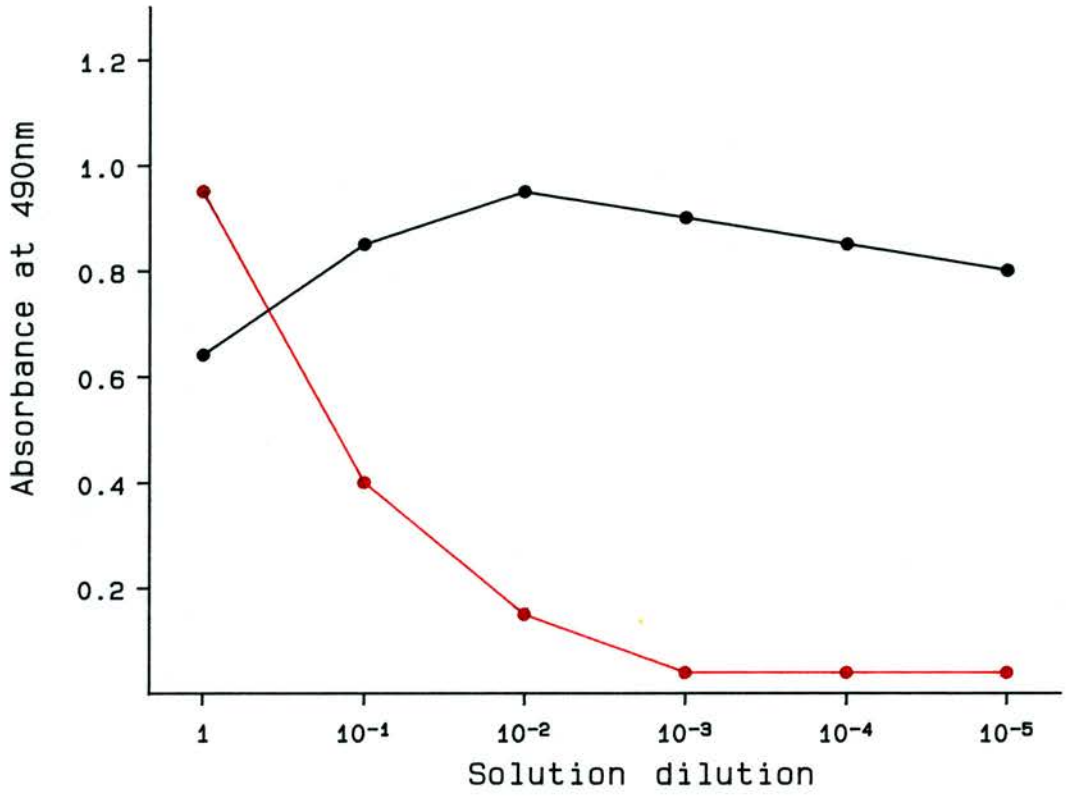

Figure 5.4: Le^a determinants detected by ELISA in eluant (red) and supernatant (black) from anti-Le^a-Sepharose beads.

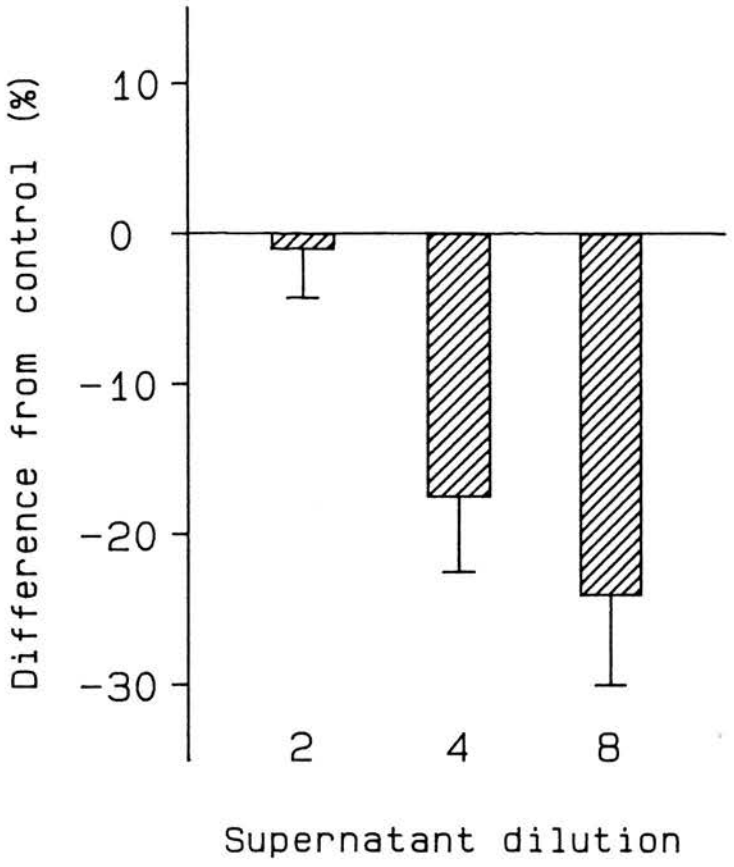

Figure 5.5: The amount of Le^a in supernatant after incubation of Le^a containing preparations with *N. meningitidis* strains. The amount of antigen in the supernatant after incubation with bacteria is shown as precentage of the positive control (antigen without bacteria). Each column represents the average of 7-13 independent observations, and the bars show ±1SEM.

5.3.4 Inhibition assay

Incubation of bacteria with preparations of purified molecules containing Le^a determinants resulted in a decrease in bacterial binding to BEC (Table 5.2). At the higher antigen concentration, a significant reduction in the mean level of attachment was found at the two ratios of BEC:bacteria tested (1:150, $p<0.05$, 1:100, $p<0.01$). When the antigen preparation was diluted 1/10 a significant decrease ($p<0.05$) in the amount of bacteria that bound to the cells was observed at the lower ratio of BEC:bacteria. When bacteria were preincubated with saliva from a non-secretor no significant decrease in the attachment to BEC from secretors or non-secretors was found at the three dilutions of saliva used (Table 5.3).

5.4 Discussion

In this chapter the hypothesis that the Le^a determinant is a receptor for *N. meningitidis* was investigated. As a first stage, an ELISA method was developed for the detection of Le^a. With this method, higher levels of Le^a determinants were found in saliva from non-secretors compared with saliva from secretors (Figure 5.3). Lewis-positive donors (both secretor and non-secretor) were found to express higher levels of Le^a then the Lewis-negative donor (Figure 5.2). These results are in agreement with the genetic control for the production of Lewis antigens described in section 1.1.3. According to this model, since the Se enzyme is not present in non-secretors, the fucosyltransferase coded for by the *Le* gene acts on precursor Type 1; and, therefore, only Le^a is produced. In contrast, both enzymes are present in secretors, and they compete for the same substance (precursor Type 1). However, the enzyme coded for by the *Se* gene is more

Table 5.2: The effect of incubation of bacteria with affinity purified preparation containing Le^a, on the attachment of bacteria to BEC from non-secretors.

BEC:bacteria (ratio)	Lewis[a] dilution	
	1/3	1/10
1:150	-12.9±4.9	-5.4±3.9
1:100	-9.6±2.15	-6.05±2.42

Attachment of bacteria with molecules containing the Le^a determinant is expressed as a percentage of the attachment of bacteria pretreated with diluent control. The figures represent the mean of 10 samples ±1SEM

Table 5.3: The effect of incubation of bacteria with saliva from non-secretor on the attachment of bacteria to BEC from secretors and non-secretors.

Cells	Saliva dilutions		
	Neat	1/10	1/100
Secretors	-8.5±9.3	-16.6±6.4	-12.27±8.1
Non-secretors	30±14	15.35±11.3	3.52±7.9

Attachment of bacteria pretreated with saliva from non-secretor is expressed as a percentage of the attachment of bacteria pretreated with buffer. The figures represent the mean of 9 samples ± 1 SEM.

efficient and most of the precursor chain is converted to H Type 1 and then by the action of the Le enzyme to Le^b. When the Le enzyme acts on the precursor structure before the Se enzyme, small amounts of Le^a determinants are produced in secretors. In secretor Lewis-negative individuals, Se is the only enzyme which fucosylates precursor Type 1, therefore H Type 1 is produced and no Lewis determinants are found.

The presence of Le^a epitopes after elution from Le^a-Sepharose beads was confirmed by the Le^a ELISA (Figure 5.4). With the affinity purification technique, molecules which contain the Le^a determinant will be specifically adsorbed onto the beads coated with anti-Le^a. These molecules were then eluted by dissociation of the antigen-antibody complex by low pH buffer. Since this determinant is present on variety of molecules (see section 1.1.3), the eluant does not contain a purified molecule, but a group of molecules all containing at least one Le^a determinant.

The expression of Lewis determinants on the surface of the BEC could not be measured (as developed for the H determinants, see section 3.2.1) due to problems with the specificity and cross-reactions of the monoclonal and polyclonal antibodies when used with BEC. However, it has been shown by immunohistochemical methods that Le^a determinants are present on BEC of non-secretor, Lewis positive individuals (Vedtofte, 1985).

In this chapter, bacteria were shown to adsorb molecules containing Le^a determinants (Figure 5.5). The amount of Le^a present in the supernatant was significantly decreased following incubation with bacteria compared with the amount of Le^a in the positive control (no bacteria). These results indicate that the bacteria have sites on their cell surface that adsorb Le^a. Up to 30% of the determinants present in the solution were adsorbed by the bacteria. Increasing

the amount of bacteria (up to 100 times) mixed with the same amount of antigen did not result in further reduction in the level of Le^a left in the supernatant (data not shown). It is possible that for some unknown reason only 30% of the Le^a determinants measured by the ELISA are available for bacterial binding.

In contrast to the inhibition of attachment observed with affinity purified preparations containing the Le^a determinant (Table 5.2), Le^a antigen in the natural soluble form, non-secretor saliva, failed to inhibit bacterial binding to non-secretors cells (Table 5.3). This suggests that this antigen might be an attachment site on epithelial cells, but its presence in secretions of non-secretors does not provide protection against colonization of the cells by these bacteria. The difference in the action of the antigens in the two preparations might be due to difference in the viscosity and/or purity. Saliva, which failed to inhibit attachment, is more viscous than the preparations obtained from the Sepharose beads. This could interfere with the ability of the Le^a antigens to interact with the bacteria.

In this chapter there are two pieces of evidence to suggest that bacteria express an adhesin that binds to molecules containing Le^a. 1) Affinity purified preparations of molecules containing Le^a inhibited bacterial attachment to BEC. 2) Following incubation with bacterial suspensions, the amount of Le^a determinants in the supernatant was reduced. Since the preparation used contains structures other than the Le^a determinants, the results presented suggest rather than prove that the Le^a determinant is a receptor.

Another possibility is that the bacteria bind to an internal structure further down the carbohydrate chain. The increased susceptibility of non-secretors could be explained if the receptor is more accessible on non-secretors' cells than on secretors' cells. It is possible that precursor Type 1 is a receptor for the bacteria.

In most secretors (90–93%), the precursor chain is di-fucosylated i.e. by the action of the Se and the Le enzymes. In the same proportion of non-secretors, only the one fucose molecule is added to the precursor Type 1 chain. If the precursor chain acts as a receptor for the bacteria, the site is probably more accessible to the bacteria in the form expressed by non-secretors. In addition, since only one enzyme fucosylates this precursor chain in non-secretors, it is possible that more precursor determinants are left un-fucosylated. It has been shown that *E. coli* and the Shiga toxin can bind to internally placed Galα1–4Galβ, present in the core structures of various molecules (for review see Bock *et al.*, 1988).

Chapter 6

Binding of glycoconjugates to outer membrane proteins of meningococci

6.1 Introduction

The binding of meningococci to epithelial cell surfaces is probably mediated by macromolecules associated with the bacterial outer membrane. Studies with human cells, cell lines and organ cultures have implicated three meningococcal surface components in attachment: pili, heat modifiable outer membrane protein (protein 5) and the major porin proteins (proteins 2 and 3).

Pili are hairlike surface appendages which radiate several micrometres from the surface of meningococci. Several studies have implicated pili as a major attachment ligand of meningococci (Salit and Morton, 1981; Stephens and McGee, 1981; Trust *et al.*, 1983) and gonococci (for a review see Stephens, 1989). Results of experiments in which gonococci were chemically modified to alter their surface charge, suggested that pili participated in the first stage of a two stage attachment process (Heckels *et al.*, 1976). It was proposed that initially pili are able to overcome the electrostatic barrier which exists between the negatively charged surfaces of the bacteria and the host cell. This increases the probability of a closer approach, leading to a stable adhesion which involves other components of the bacterial surface including outer membrane proteins. This second stage may occur even when the cell is not pilate. The precise mechanism by which pili are able to overcome the electrostatic barrier is not clear. One possibility suggested by Heckels (1989) is that because of their small surface area, pili are less sensitive to the electrostatic repulsive forces than the surface of the bacteria. Pili of meningococci are difficult to maintain and are lost on serial transfer *in vitro* (Salit and Morton, 1981).

The meningococcal outer membrane contains from two to five major proteins when examined by SDS-PAGE. They can be catagorized into five different

classes according to their apparent molecular weight on SDS-PAGE (for a review see Frasch *et al.*, 1985). These five major outer membrane proteins can be grouped into two classes on the basis of their reaction to heat and solubility in detergents (Frasch and Mocca, 1978). Protein 5 of meningococci was defined as heat modified protein which is similar biochemically to the heat modified protein of gonococci (P2). For gonococci, several studies have indicated the importance of P2 as an attachment ligand (for a review see Stephens, 1989). The importance of protein 5 in mediating attachment of meningococci is less well established. Initial reports suggest that this protein is a ligand involved in meningococcal attachment (Stephens, 1989).

A role for the major porin proteins (protein 2 and 3) in attachment and invasion was proposed by Lynch *et al.* (1984). These workers reported that the porin molecules of gonococci and meningococci interact with a lipid bilayer. It was proposed that during this process a channel is formed through the lipid bilayer, that could aid the attachment and invasion process.

In this chapter, an attempt was made to identify the structures on the bacteria and on the carbohydrate chains of the blood group determinants which interact in a lectin-like manner. Synthetic glycoconjugates carrying blood group determinants with defined structures were used in an ELISA assay. This method was used to investigate the interaction of blood group determinants with outer membrane proteins (OMPs), extracted from *N. meningitidis* strain C:2b:P1.2.

6.2 Materials and methods

6.2.1 Examination of bacteria for pili

The presence of pili on the bacterial surface was examined by negative staining. A sample of fixed *N. meningitidis* (C:2b:P1.2) was stained with potassium phosphotungstate (PTA) by the method described in Hayat (1989).

6.2.2 Preparation of outer membrane proteins

N. meningitis (C:2b:P1.2) was grown overnight on MNYC agar in a humidified atmosphere with 10% CO_2 at 37°C. Bacteria were harvested into 60 ml of PBS (pH 7.2) to give a heavy suspension and centrifuged at 2000 g for 20 min. The bacteria were resuspended in 30 ml of PBS; 6 ml at a time were sonicated on ice, three times, at an amplitude of 6 microns for 50 sec (MSE, Leicestershire, UK). Unbroken cells were removed by centrifugation at 3000 g for 20 min at 4°C; and, the total cell envelope fraction from the supernatant was pelleted by centrifugation at 60,000 g at 4°C for 60 min. The pellet was resuspended in 30 ml of 2% (w/v) sodium N-lauroylsarcosine ('Sarkosyl', Sigma) for 20 min at room temperature. This detergent selectively solubilizes the inner membrane from the crude envelope preparations and provides a method for examining the outer membrane (Hancock and Poxton, 1988). The remaining outer membrane fraction was pelleted by centrifugation at 60,000 g at 4°C for 60 min, resuspended in distilled water and stored at –20°C.

6.2.3 Protein estimation

Protein concentration was determined using the Coomassie brilliant blue assay (Bradford, 1976).

6.2.4 SDS-PAGE

Proteins were separated by SDS-PAGE using the SDS-discontinuous system of Laemmli (1970) on a mini-protean II cell (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK). Equal volumes of protein sample and sample buffer were mixed and heated to 100°C for 3 min. Approximately 20 μg of protein were applied to each lane and electrophoresis performed at a constant voltage of 100V through the stacking gel (5% acrylamide) and at a constant voltage of 150V through the resolving gel (10% acrylamide). Proteins were visualised by staining with Coomassie blue (0.5% (w/v) Commassie brilliant blue in 25% (v/v) propan-2-ol + 10% (v/v) glacial acetic acid) followed by destaining with 10% (v/v) glacial acetic acid. Molecular weight markers (Sigma) in the range of 26,600 to 180,000 were run in parallel. The proteins were identified by their molecular weight as defined in Frasch *et al.*, 1985.

6.2.5 Binding of Type 1 blood group determinants to OMPs

The composition of buffers used in this assay are given in section 2.6. Blocking buffer and washing buffer given in that section were modified by using gelatin instead of BSA.

The wells of polystyrene microtitre plate (M129B, Dynatech, Bellinghurst, Sussex, UK) were coated overnight at 4°C, with 100 μl of 300 μg/ml OMPs in coating buffer. All further procedures were carried out at room temperature. The wells were washed three times with washing buffer (0.03% gelatin) then blocked for 60 min with blocking buffer (0.3% gelatin). The blocking buffer was removed and the wells were washed twice with washing buffer. Fifty microlitres of sugars conjugated to human serum albumin (HSA) obtained from BioCarb (Russell Fine Chemicals, Chester, UK) or HSA (cat no. A3782, Sigma) diluted in blocking buffer (10–50 μg per well) were added to each well for 120 min. The synthetic glycoconjugates used were Lacto-N-tetraose (precursor Type 1), Lacto-N-fucopentaose I (H Type 1), Lacto-N-fucopentaose II (Lewis^a) and Lacto-N-difucohexaose I (Lewis^b). After 3 washes, 100 μl of 1/100 dilution of biotinylated polyclonal chicken anti-HSA (Sera-lab LTD, Sussex, UK) in blocking buffer were added and incubated for 60 min. Unbound antibody was removed by washing three times; and, 100 μl of 1/100 dilution of streptavidin biotinylated horseradish peroxidase complex (Amersham, Amersham, UK) in blocking buffer were added for 30 min. After three washes, 100 μl of substrate solution were added. The reaction was allowed to develop in the dark for 20 min and stopped by adding 50 μl of 12.5% (v/v) H_2SO_4. The absorbance at 490 nm was measured with a Dynatech plate reader.

6.2.6 The influence of coating the bottom of the well with different proteins on the binding of HSA-precursor Type 1

The wells of a microtitre plate were coated overnight at 4°C with 100 μl of either OMPs, human transferrin, insulin (bovine pancrease), chicken egg albumin

(grade V) or rabbit albumin (all 50 μg per well) in coating buffer. The last four proteins were purchased from Sigma. All further procedures were carried out at room temperature. The wells were washed three times, blocked for 60 min and washed twice. Fifty microlitres of Lacto-N-tetraose (precursor Type 1) conjugated to HSA, HSA diluted in blocking buffer (25 μg per well) or diluting buffer were added to each well for 120 min. The rest of the assay was carried out as described in section 6.2.5.

6.2.7 The effect of free sugar on the binding of blood group determinants to OMPs

The assays used to study the specificity of the binding of the blood group determinants to the OMPs, are based on the ELISA system described in section 6.2.5. Two assays were used:

Assay 1:

The wells of microtiter plate were coated with OMPs. After washing and blocking, 25 μl of 10 mM, 5 mM or 1 mM Lacto-N-tetraose (precursor Type 1) carbohydrate (BioCarb) (diluted in blocking buffer) or blocking buffer were added to each well. After 60 min incubation, 25 μl of Lacto-N-tetraose (precursor Type 1) conjugated to HSA (BioCarb) or HSA (Sigma) diluted in blocking buffer (25 μg per well), as the negative control, were added directly to each well for 120 min. Detection of bound HSA was carried out as described in section 6.2.5.

Assay 2:

In this assay, after 1 hr incubation with the free carbohydrate (5 mM, 1 mM or 0.5 mM), unbound sugar was removed by washing twice in washing buffer. Then, 50 μl of precursor Type 1 conjugated to HSA (BioCarb) or HSA (Sigma) diluted in blocking buffer (25 μg per well), as the negative control, were added to each well for 120 min. Detection of bound HSA was carried out as described in section 6.2.5.

6.3 Results

6.3.1 Electron microscopy of meningococci

Electron microscopy showed that pili were not present on the surface of the bacteria used in these experiments (Figure 6.1).

6.3.2 OMP profile obtained on SDS-PAGE

SDS-PAGE analysis of the crude 'Sarkosyl' extract produced a profile typical of Gram-negative OMPs; there were four to five major OMPs and a further 15–25 minor proteins (Figure 6.2). The three major outer membrane proteins identified in this preparation are 1, 2 and 5 characterized by their apparent molecular weights on SDS-PAGE of 46,000±1,000, 41,000±1,000 and 28,000±1,000 respectively. These results are in accordance with the type of bacteria used indicating the presence of a protein 2 and a protein 1.

Figure 6.1: Electron micrograph showing *N. meningitidis* C:2b:P1.2.

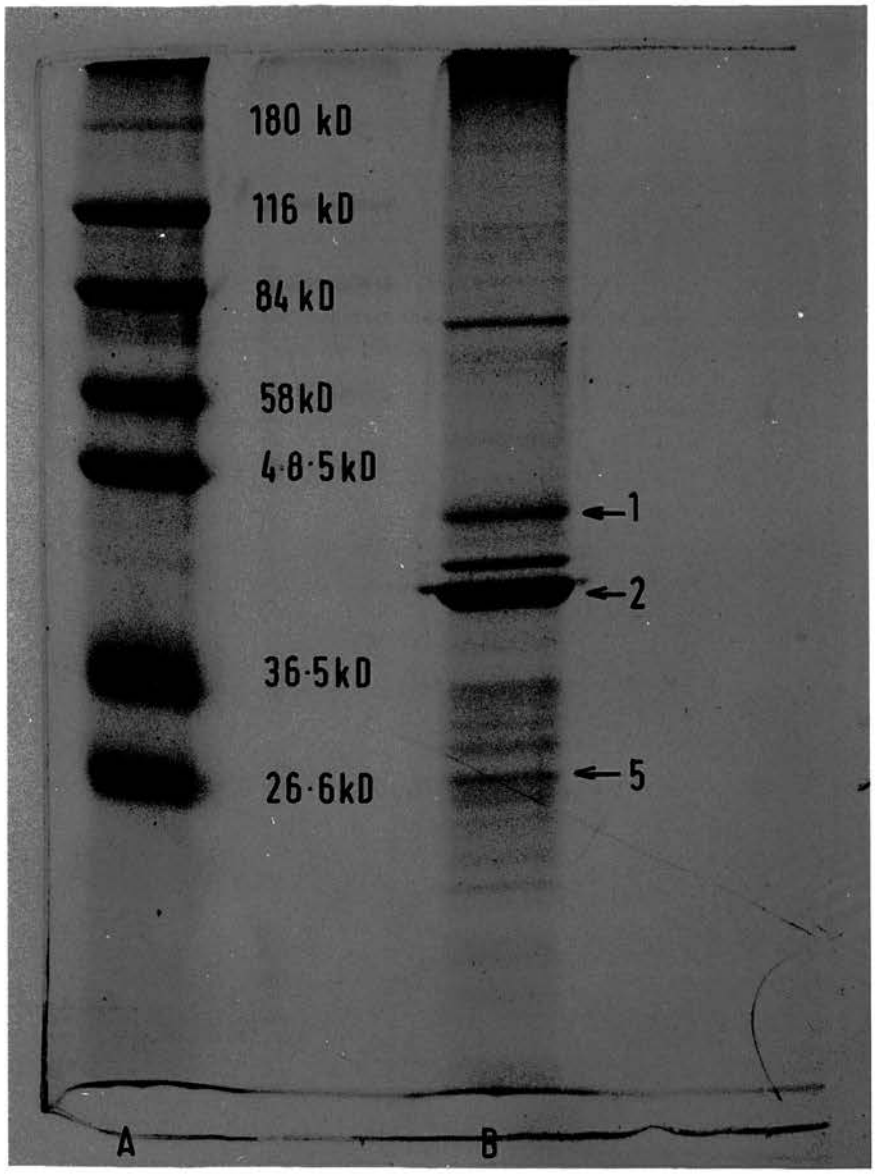

Figure 6.2: Separation of outer membrane preparation of *N. meningitidis* (C:2b:P1.2) by SDS-PAGE in a 10% gel stained with Coomassie brilliant blue. The molecular weight markers in lane A and the OMPs in lane B. The identity of the major outer membrane proteins are shown.

6.3.3 Binding of blood group determinants to OMPs

Several synthetic glycoconjugates were used to study the binding of blood group determinants to OMPs in an ELISA system. The antigens are oligosaccharides purified from human milk and attached to HSA via a spacer (acetylphenylenediamine). The conjugates contain 10–20 molecules of oligosaccharide per molecule of HSA. As shown in Table 6.1, all the glycoconjugates tested bound to the OMP; in contrast, HSA did not bind to the OMP under the same conditions. The binding of the different determinants could not be compared, since the amount of oligosaccharides per HSA molecule is different in each preparation. Since the precursor chain is the common structure in all the glycoconjugates examined, it was used in the following experiments.

6.3.4 The influence of glycoconjugate concentration on binding to OMPs

The binding of precursor conjugated to HSA to OMPs was dependent on the amount of glycoconjugate added (Figure 6.3). Maximum binding was reached when 40 μg of antigen per well were used. A point around the middle of the slope (25 μg per well) was used in the following assays.

6.3.5 Binding of a glycoconjugate to different proteins

The ability of precursor-HSA to bind to several proteins in the ELISA system was examined, to determine whether the binding of this glycoconjugate is specific to OMPs. As shown in Figure 6.4, with the glycoconjugate, positive readings

Table 6.1: Binding of glycoconjugates to outer membrane proteins of *N. meningitidis*

	O.D.
HSA	0.020
HSA-Precursor Type 1	0.495
HSA-H Type 1	0.426
HSA-Lewis^a	0.356
HSA-Lewis^b	0.597

The binding of human serum albumin (HSA) and HSA conjugated with blood group determinants to OMPs of meningococci. The results are expressed as optical density (O.D) as measured by an ELISA plate reader at 490 nm.

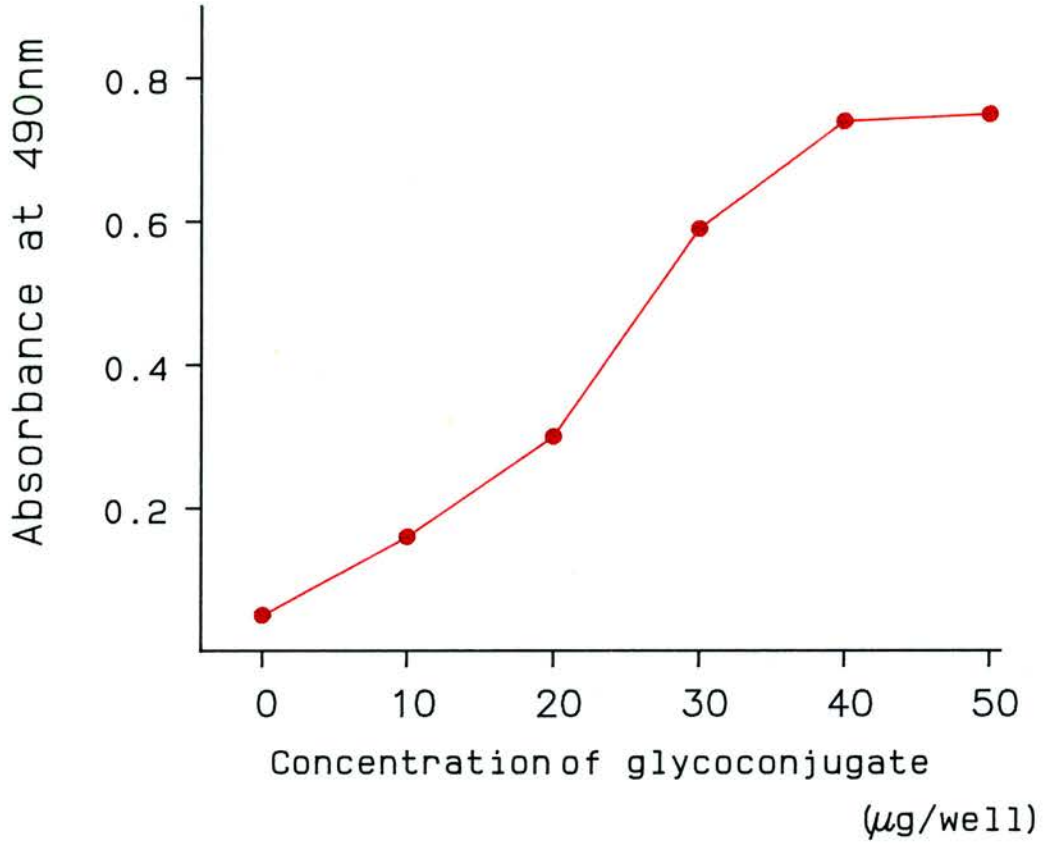

Figure 6.3: Binding of precursor-HSA glycoconjugate to OMPs of *N. meningitidis*. Concentration '0' indicates the presence of unconjugated HSA at 50 μg/well.

were found for three of five proteins tested: the OMPs, transferrin and rabbit albumin. For the last two, positive readings were obtained for the two controls, which indicates that the anti-HSA used probably cross-reacts with the proteins on the bottom of the plate. In contrast, the negative controls for the OMPs were both negative, indicating that the positive reading obtained is due to a specific binding of the glycoconjugate to the OMPs.

6.3.6 The influence of free oligosaccharide on the binding of glycoconjugate to the OMPs

The precursor Type 1 oligosaccharide was used to study the effect of free oligosaccharide on the binding of the same oligosaccharide conjugated to HSA. When the free saccharide was present during the incubation of the glycoconjugate with the OMPs an increase was found in the amount of glycoconjugate bound (Figure 6.5a). This increase was directly proportional to the amount of saccharide added up to 5 mM. If unbound saccharide was removed before the addition of the glycoconjugate then no apparent effect on the binding of the glycoconjugate was found (Figure 6.5b).

6.4 Discussion

In this chapter an attempt was made to characterize the meningococcal surface adhesins and the structure in the blood group determinants which might be a receptor on the host cell. The outer membrane proteins were extracted from the strain of *N. meningitidis* used in the previous chapters. As indicated by the electron micrograph, this strain is nonpilate (Figure 6.1). Therefore, structures

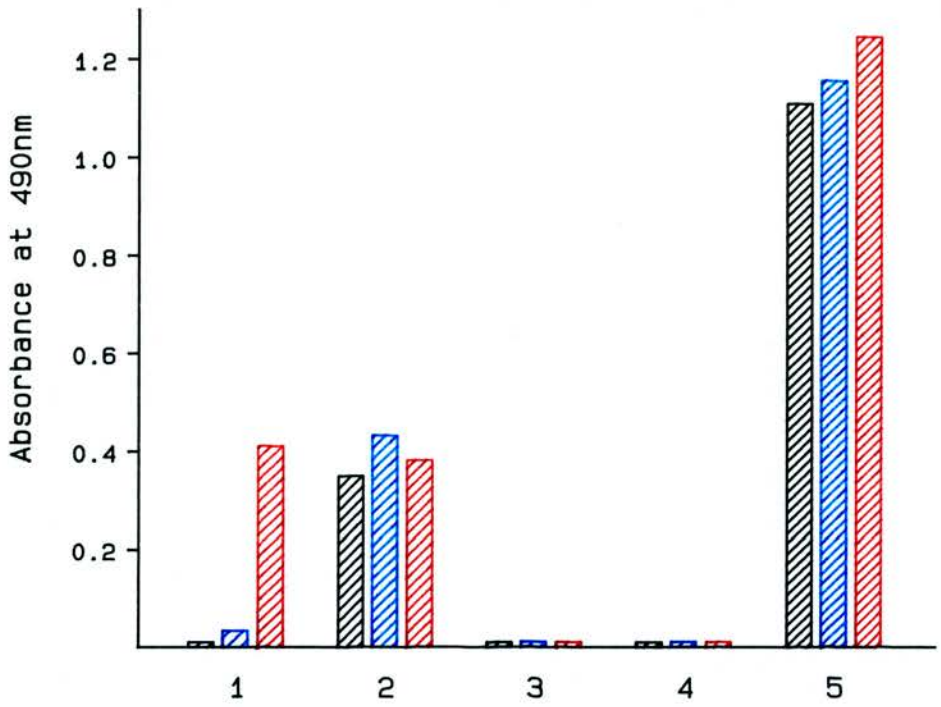

Figure 6.4: The binding of precursor-HSA glycoconjugate (detected by anti-HSA) to OMPs of *N. meningitidis* (1), human transferrin (2), bovine insulin (3), chicken albumin (4) and rabbit albumin (5). The red column is the reading in the presence of precursor-HSA glycoconjugate. The HSA and PBS controls are shown by the blue and black columns respectively.

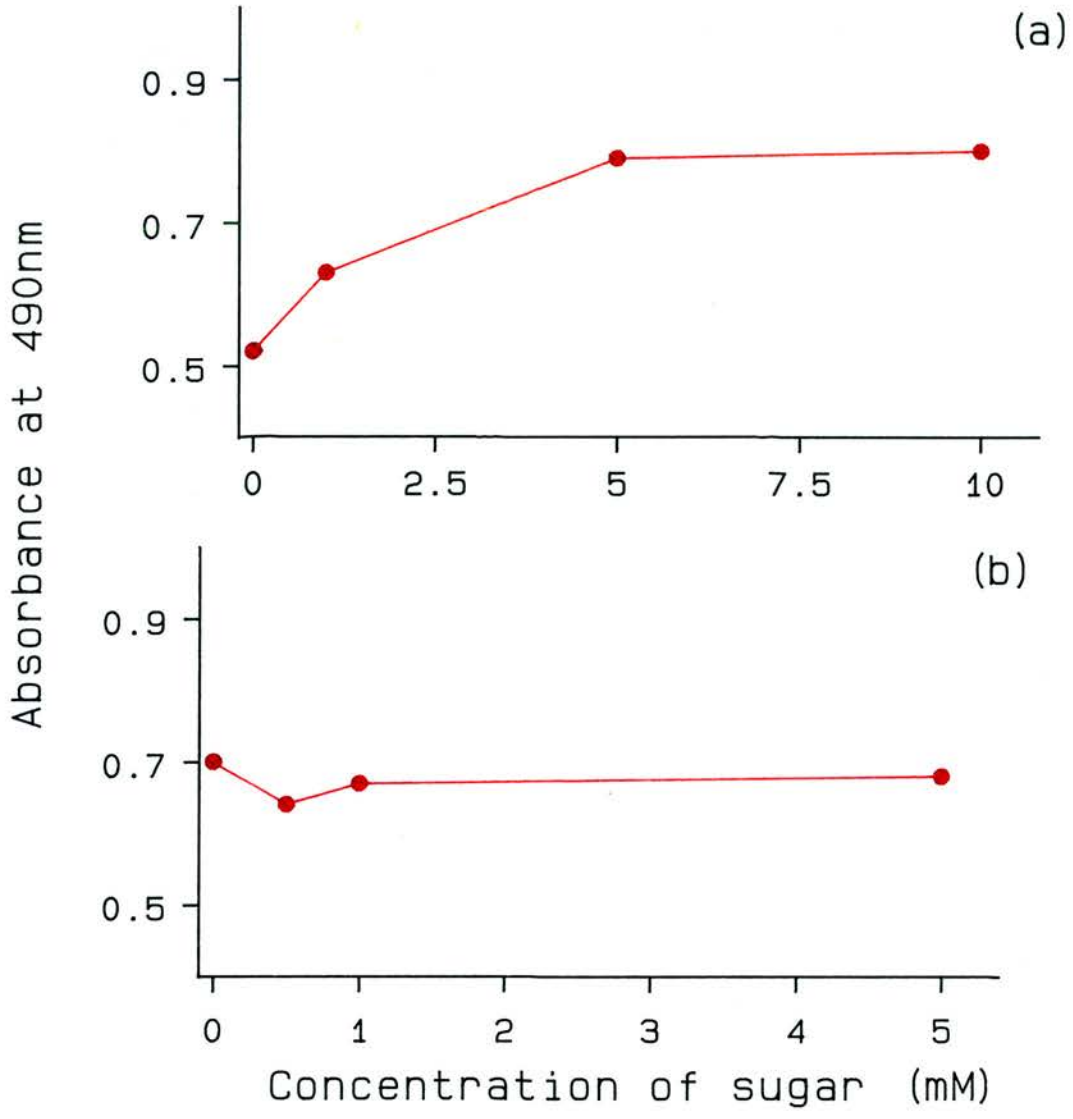

Figure 6.5: The effect of the oligosaccharide on the binding of precursor-HSA glycoconjugate to OMPs: (a) in the presence of oligosaccharide during the binding and (b) when unbound oligosaccharide was removed before the addition of glycoconjugate. The figure presents values obtained for one of three experiments.

other then pili on the bacterial outer membrane must be considered, to explain the results of the previous chapters which suggested a role for Le^a in bacterial binding.

The importance of OMPs other than pili in the attachment of gonococci and meningococci has been demonstrated in several studies. More information is available on these interactions for gonococci than meningococci. In a recent report, *in vitro* studies indicated that both pilate and nonpilate gonococci adhere to and were internalized by tissue culture cells with equal efficiency (Shaw and Falkow, 1988). In another study, a set of glycolipid receptors on epithelial cells that are recognized by both pilate and nonpilate variants of gonococci have been identified (Deal *et al.*, 1988). These glycolipids share lactose as their core sugar. Binding to these structures was demonstrated to be independent of pili and protein 2 expression. The bacterial gene that codes for the ligand was recently identified and the ligand was observed to be a 36 kD surface protein not associated with the pilus (Paruchuri *et al.*, 1990). Very little is known about the role of OMPs of meningococci other than pili in mediating attachment to host cells. However, the role of these proteins is not in doubt since attachment was demonstrated in nonpilate bacteria as well as pilate variants. A role for the heat modifiable protein (protein 5) was reported for urogenital meningococci strains (Hugman and Danielsson, 1989). They observed that these strains attached significantly more efficiently to vaginal epithelial cells in the presence of the heat modifiable proteins.

In this study the OMPs extracted from meningococci and attached to a solid matrix were found to bind synthetic glycoconjugates which carry Type 1 blood group structures. The importance of the presence of the carbohydrate chain for this interaction is indicated by the negative result obtained when HSA without

a saccharide moiety was added.

When the binding of the precursor-HSA to proteins, other than OMPs', attached to the solid matrix was examined, two proteins showed positive interaction (Figure 6.4). This interaction was not specific, as indicated by the negative controls which gave similar readings as the test. These results were not unexpected since transferrin is a human serum protein which might have been injected into the chicken as a contaminant of the HSA; and albumin from the rabbit is probably similar to human albumin since both are mammals.

From these results it is proposed that the carbohydrate moiety is the structure on the glycoconjugate that binds to the OMPs in the ELISA system. The common structure on all glycoconjugates tested is the Type 1 precursor chain, therefore, this structure was used in additional assays. The free precursor Type 1 oligosaccharide failed to inhibit the binding of precursor Type 1 glycoconjugate to the OMPs. In fact, an increase in the binding of the glycoconjugate was found in the presence of the oligosaccharide. This effect resembles an allosteric effect for enzymes in which an enzyme possesses at least two spatially distinct binding sites on the protein molecule: the active site and the regulatory site. Binding of a molecule to the regulatory site produces a change in the active conformation so that activity at this site may be increased (see Newsholme and Start, 1974). In our system, it might be that the protein in the OMPs preparation which binds the glycoconjugate has an additional site which binds free saccharide. Binding of the free saccharide might cause a conformational change that allows higher affinity binding of the glycoconjugate. An increase in the glycoconjugate binding to the OMPs was not demonstrated when the free oligosaccharide was removed before the HSA-precursor was added. This could be due to low affinity binding of the free saccharide to the OMPs. To further examine the specificity of this

effect monosaccharides and oligosaccharides other than precursor Type 1 chain need to be examined in this system.

Chapter 7

General discussion

Detailed discussion of the results has been given at the end of each chapter. The objective of this chapter is to provide more general conclusion to this study, point out limitations in the experimental methods used and prospects for further research.

Non-secretors of ABH blood group determinants are more susceptible to several infectious diseases including meningococcal meningitis and are over-represented among carriers of *N. meningitidis* (see section 1.2.3 and 1.2.4). The mechanisms underlying these observations are not known. In this study an attempt was made to identify some of the interactions between host and parasite that contribute to these epidemiological observations. The study is based on hypotheses proposed to explain the association between non-secretion of blood group antigens and susceptibility to infectious diseases. Both hypotheses suggest that differences between secretors and non-secretors in the expression of carbohydrate structures on their epithelial cells result in differences in the degree of colonization. These hypotheses are supported by increasing evidence that carbohydrate structures on cell surfaces act as specific receptors for bacteria (see section 1.4.3). The epidemiological evidence indicates that non-secretors are over-represented among patients compared with their proportion in the control populations. However, individuals of both phenotypes, i.e. secretors and non-secretors, are found among patients, and the proportion of non-secretors with the disease is not necessarily higher than that of secretors with the disease. This fact suggests that there are additional mechanisms operating in non-secretors that make them more susceptible to disease, or that non-secretors are deficient in a defence mechanism. Since both populations are susceptible to infections by *N. meningitidis*, examination of one aspect of the bacterial pathogenesis or the host immune mechanisms can only be expected to show minor differences between individuals from the two populations.

Two structures were investigated in this study as possible receptors for bacterial attachment on the surface of epithelial cells. The H determinant was suggested to be a receptor on the cells of both secretors and non-secretors. The presence of this determinant in the secretions of secretors could act as an innate defence mechanism; the antigen in secretions might bind to adhesin on the bacteria and reduce colonization of cells of secretors. This hypothesis assumes that secretors and non-secretors are similar with respect to the presence and level of H determinants on the cell surface. To confirm this assumption, the first stage in the investigation was to determine the level of the H determinant on epithelial cells of secretors and non-secretors. In contradiction to the assumption made by the hypothesis, H was found to be expressed in higher levels on various epithelial cells from secretors compared with cells from non-secretors (see Figure 3.5). In subsequent experiments, bacterial attachment to BEC from secretors and non-secretors was compared. The results obtained did not positively correlate with the expression of H determinants on the cells. These results rule out H as a possible receptor for meningococcal attachment.

Le^a is another structure suggested to act as a receptor for bacterial attachment on epithelial cells. This determinant is present at higher levels on cells of non-secretors; and, if it acts as a binding site, should result in greater bacterial attachment to cells of non-secretors compared with those of secretors. For the reasons described in section 4.4, the methods available for studying bacterial attachment to host cells were not used. Instead, a flow cytometric assay was developed to measure bacterial binding.

7.1 Analysis of bacterial attachment by flow cytometry

The attachment of bacteria to BEC from secretors and non-secretors was compared by flow cytometry. By using this method most of the disadvantages described for attachment assays were resolved (see section 4.4); however, variations between individuals in the expression of blood group determinants and other factors were difficult to control. In addition, individuals from both groups are susceptible to colonization by bacteria. Thus, if a blood group determinant that is present solely on cells of one population is involved in attachment, it must only be part of the repertoire of receptors available for bacterial binding. The high variation between individuals and the involvement of more than one receptor decreases the chances of finding significant differences in bacterial binding between secretors and non-secretors. It is encouraging that the differences obtained by the flow cytometric assay, though not significant at the 95% confidence level, are significant at the 90% confidence level. Taking into account the above limitations, the differences observed in attachment strongly suggests, but do not prove, that cells of non-secretors bind more bacteria.

There are several limitations to the use of flow cytometric analysis of bacterial attachment to epithelial cells. A higher level of binding to non-secretors' cells compared to secretors' cells was demonstrated when low ratios of bacteria per cell were used. A further decrease in the ratio of bacteria per cell might have resulted in statistically significant differences between the two populations. In order to distinguish between positive and negative cells with respect to bacterial binding, there is a need for a certain differential level of fluorescence on the cell surface. If more than one bacterium has to bind to obtain a positive reading,

then increasing the intensity of fluorescence might allow the use of lower ratios of bacteria per cell. However, the sensitivity of the flow cytometer given the fluorescent dye used, did not allow a further decrease in the ratio of bacteria per cell.

A further limitation is the inability to express the binding of bacteria to cells in terms of numbers of bacteria per cell. An attempt was made to determine the number of bacteria attached to each cell. This information can be obtained if the mean fluorescence level for one bacterium can be determined. The mean fluorescence level on the cells (on a linear scale) is directly proportional to the number of bacteria attached. In principle it should be simple to measure the fluorescence intensity of a single bacterium; but, the small size of organisms and problems with aggregation made this difficult to perform. Improvements in the instrumentation (e.g. diameter of the tip's orifice) or treatment of bacteria to stop agglutination might help to solve some of these problems.

Although the number of bacteria bound to cells is not known, the attachment index used implies that more bacteria bind to the cell populations obtained from non-secretors compared with those obtained from secretors. Higher level of binding to non-secretors cells can be explained by: 1) more receptors available on these cells for specific interactions or 2) higher affinity interactions between binding sites on non-secretors cells and an adhesin on the bacteria. In the first case, Le^a can be suggested as a receptor on the cells, since this antigen is expressed in higher levels on cells of non-secretors compared with cells from secretors (see section 1.1.3). In the second, the inner carbohydrate chain of the Type 1 blood group antigens (the precursor Type 1 chain) could be suggested as a receptor. Differences between secretors and non-secretors in the level of glycosylation of the Type 1 precursor structure might result in differences in

its accessibility for bacterial adhesins. This in turn could effect the affinity of interactions with this receptor. In both cases, carbohydrate structures on the cells are suggested to act as receptors for bacterial binding. The specificity of the interaction between the bacteria and a carbohydrate on the host cell might be demonstrated by inhibition of attachment.

7.2 Inhibition of attachment

Two main methods have been used in previous studies to determine the carbohydrate structure on the host cell involved in bacterial attachment: 1) blocking the proposed binding sites on the cells with specific antibodies directed against the antigen that acts as a receptor; and 2) inhibiting the interaction by blocking the bacterial ligand with a large excess of sugars that are thought to compose the carbohydrate receptor on the host.

Blocking bacterial binding by antibodies

Monoclonal antibodies against the Le^a determinants were tested for their ability to block bacterial binding, by treatment of BEC with these antibodies prior to incubation with the bacteria (data not shown). The antibody failed to inhibit bacterial binding. Enhancement in the binding of bacteria was observed probably due to non-specific interactions between the bacteria and the antibody. In addition, the specificity of the anti-Le^a when applied to BEC is in doubt.

Furthermore, it has been argued that inhibition of attachment by antibodies does not necessarily identify the receptor on the cells (Freter, 1981). In the

discussion following his lecture, Freter suggested that antibodies might block attachment by steric hindrance or by other non-specific mechanisms. Hence, inhibition of attachment by specific antibodies cannot be used to identify the epitope used as a receptor in bacterial attachment. On the other hand, no inhibition of attachment when cells are treated with antibodies indicate that the epitope is not the structure to which the bacteria bind.

Sugar inhibition of attachment

Sugar inhibition studies were successful in the classical case of mannose binding bacteria (Ofek *et al.*, 1977) and a number of carbohydrate receptors have been discovered using this approach (Beachey, 1980). In the present study, a wide range of monosaccharides failed to inhibit attachment (data not shown). This phenomenon has been reported elsewhere and discussed by Bock *et al.* (1988). In this review, it was suggested that soluble univalent oligosaccharides for attachment inhibition might inhibit attachment only for binding sites with relatively high affinity. When the binding is of low affinity, experiments might produce misleading negative results. Inhibitory substance might be unable to compete with binding mediated by the presence of multiple receptors on the cell surface. An example of this is the inability to inhibit the specific interaction between Shiga toxin, produced by *Shigella dysenteriae* type 1, and Galα1–4Gal by a high concentration of free disaccharide. In contrast, the disaccharide coupled multivalently to bovine serum albumin gave a 50% inhibition at a low level of saccharide. The use of multivalent glycoconjugates (glycoproteins or glycolipids) might result in micellar formation which is difficult to control (Bock *et al.*, 1988). This in turn might produce false positive or false negative results depending on exposure of hydrophobic surfaces or masking of binding epitopes.

Inhibition of meningococcal attachment to BEC

In the experiments described in chapter 5, affinity purified preparations containing Le^a determinants were used for inhibition of meningococcal attachment. Although the Le^a determinants in these preparations are probably in the multivalent form, the results, though statistically significant, did not indicate a high level of inhibition (12.9±4.9%). The results do not conclusively indicate a role for Le^a determinants in the attachment of *N. meningitidis*. Another major problem with the interpretation of these results is the impurity of the preparations used. The method used for obtaining these preparations selects for antigens containing Le^a determinants as part of the molecule. The backbone molecules which express these determinants will be different and the effect of the other structures on bacterial attachment cannot be ruled out. Only late in the study did the antigens being investigated become commercially available in a pure form. Due to their high cost, they could only be used in a limited number of experiments. The inability to inhibit bacterial attachment with free saccharides while achieving inhibition with preparations containing Le^a, suggests that if Le^a determinants are involved in specific interactions with bacteria they are of a low affinity. It has been proposed that low affinity binding might have been selected for in some ecological niches to avoid inhibition by free oligosaccharides (Bock *et al.*, 1988). Free oligosaccharides, including blood group structures, are known to be present in secretions. They may also be present at the target cell surface as a result of physiological enzymatic degradation (Bock *et al.*, 1988).

Bacterial attachment was inhibited by partially purified preparations containing Le^a determinants (Table 5.2). The high levels of Le^a in secretions of non-secretors (see figure 5.3) raises the possibility that this determinant in mucus could block attachment to epithelial cells, thereby reducing colonization of

non-secretor cells *in vivo*. However, saliva from a non-secretor donor failed to inhibit meningococcal attachment to BEC (see Table 5.3). This may indicate that if Le^a is a receptor, then the bacteria cannot interact with this structure in secretions (see section 5.4). Even if it does interact with Le^a in secretions, it should be stressed that, although association with receptors in mucus is generally regarded as a mechanism that reduces bacterial colonization, this need not always be the case. An alternative possibility will be discussed in the following section.

7.3 The role of mucus in bacterial colonization

Mucus is a secretion that covers most epithelial cell surfaces. It consists of very large and complex glycoproteins (mucins), in which the carbohydrate fraction is 70%–90% of the dry weight. On some mucosal surfaces, such as those of the gastro-intestinal tract and respiratory tact, the mucus is thought to form a major barrier against penetration of bacteria to the level of the epithelial cells. Adherence of bacteria to the mucus layer overlying the epithelial cells might be to the advantage of the host in the presence of a functioning mucociliary clearance system.

The bronchial tree is protected against inhaled particles, including bacteria, by the mucociliary system. This is composed of a mucus layer covering cilia which are hair-like structures. Inhaled particles stick to the mucus surface and are moved toward the back of the throat by the coordinated beating of the cilia. If this system malfunctions, then trapping of bacteria in the mucus gel may be the first step towards colonization. In the case of *N. meningitidis*, damage to ciliary activity was noted early in the course of infection of human

nasopharyngeal tissue in organ culture (Stephens *et al.*, 1987). The damage to the ciliated cells was not associated with the attachment of meningococci to these cells. It has been suggested that *N. meningitidis* damages ciliated epithelial cells indirectly by release of lipopolysaccharides (LPS), peptidoglycan monomers or possibly other toxins from the bacteria. In addition, bacterial infections of the lower respiratory tract often occur following viral infection (see Wilson and Moxon, 1988). Several mechanisms have been suggested to explain potentiation of bacterial infection by viruses including loss of ciliated epithelium (Wilson *et al.*, 1987). Exposure to cigarette smoke is one of the environmental factors associated with carriage of *N. meningitidis* (Blackwell *et al.*, 1990) and damage of cilia activity (see Wanner, 1990). Thus, in meningococcal infection, if the ciliary activity has been damaged by the release of endo-toxins, viral infection or by passive or active exposure to cigarette smoke, binding of the bacteria to the mucus layer could be an advantage to the bacteria. Thus, specific interaction between *N. meningitidis* and Le^a determinants in mucus of non-secretors might enhance colonization.

7.4 Adsorption of antigens by meningococci

Affinity purified preparation containing the Le^a determinants were used in another assay in which the presence of binding sites for Le^a on the bacteria was examined (see section 5.3.3). In this study a reduction in the amount of Le^a determinants present in solution was found after incubation with bacteria. These results are difficult to interpret since, as discussed above, the preparation is not homogeneous with respect to its chemical make up. However, when a similar test was performed with a preparation containing H determinants (prepared from saliva of a secretor Lewis-negative individual), no decrease in the amount of

antigen detected in solution was found (data not presented). Since the source of antigens is similar (saliva), the molecules present in the Le^a and H preparations should be similar apart from the presence of these blood group determinants as part of the macromolecules. The results suggest that the bacteria have a receptor that can bind to the Le^a epitope but not to H. These results are in agreement with those obtained in the attachment study (section 4.4). Direct detection of the Le^a epitopes on the bacteria could confirm specific binding. However, detection of bound Le^a by monoclonal antibody was not attempted because the paratope and the bacteria would be competing for the same site on the blood group antigen. In addition, detection of the Le^a determinant by antibodies will not serve as direct evidence that it acts as a binding site, since the bacteria could bind through another structure on the same macromolecule.

The inability of bacteria to adsorb more than 30% of the antigen present in the solution, even with increased amounts of bacteria, is difficult to explain. It is possible that for some reason only 30% of the Le^a determinants measured by ELISA are available for bacterial binding. This could happen if 30% of the antigens detected are in the glycolipid form and the rest are glycoproteins or vice versa. If the bacterial binding site is influenced by the molecule carrying the carbohydrate chain, it is possible that the bacteria is limited to binding to glycolipids. In this case only 30% of the determinants detected by the ELISA could be adsorbed by the bacteria. The involvement of the carrier molecule in the bacterial binding site have been suggested for *E. coli* attachment to antigens with blood group determinants (Rosenstein *et al.*, 1988). In this study free carbohydrate structures failed to inhibit bacterial binding to glycoconjugate carrying the same carbohydrate chain. The authors, therefore, suggested that the adhesive specificity involves both the saccharide and lipid moiety.

7.5 Precursor chain as a possible receptor

As was suggested in section 5.4, precursor Type 1 might act as a receptor for bacterial binding. This structure is less fucosylated in non-secretors compared with secretors; and therefore, it might be more accessible in non-secretors for a bacterial adhesin. Differences in the strength of bacterial binding to glycoconjugates with different blood group determinants on a Type 1 precursor chain have been reported (Rosenstein *et al.*, 1988). Modification of the precursor structure by addition of monosaccharide (in H and Le^b determinants) interfered with binding of *E. coli*. Thus, Rosenstein *et al.* (1988) predicted that the *secretor* gene and the blood group genes that code for glycosyltransferases would influence bacterial binding and hence susceptibility to colonization. In experiments performed in this study, precursor Type 1 conjugated to HSA bound to OMPs obtained from one strain of *N. meningitidis* (section 6.3.3).

If the precursor chain is a receptor for bacteria, then a form of defence mechanism will be to produce glycosyltransferases that will modify this structure, thereby interfering with bacterial attachment to the receptor. The induction of fucosyltransferase activity a few days after conventionalization of germ-free mice has been reported (Umesaki *et al.*, 1982). On the other hand, production of enzymes by the bacteria that can degrade monosaccharides on the extended precursor chain might be a virulence factor facilitating colonization. Evidence for this process has been reported by Larson *et al.* (1987), who studied the glycosphingolipids of meconium (the first sterile stool of newborns) and faeces of newborns and young children. In this study they found that ABH blood group activity rapidly diminished after the first few days of life parallel to bacterial colonization of the intestines. The pattern of undegraded glycosphingolipids seen in meconium was found to be replaced mainly by the precursor chain (lac-

tosylceramide).

Binding to an inner structure rather than to the terminal part of a carbohydrate chain has been reported for *E. coli* and Shiga toxin. The binding site, identified as Galα1–4Galβ, is situated in the inner core of the carbohydrate chain (for a review see Bock *et al.*, 1988). Binding of microorganisms to internal structures might have been selected for in evolution to avoid variations in the terminal parts of the carbohydrate chain, as in blood group determinants.

7.6 A new hypothesis to explain differential susceptibility dependent on secretor status

Degradation of carbohydrate structures described in the previous section might have an additional function in supplying growth substances for the bacteria. If the bacterial enzymes have a higher affinity for blood group determinants found in non-secretors (i.e. Le^a) than for determinants expressed in secretors (Le^b), this suggests two susceptibility factors. The first is that non-secretors could provide the bacteria with a better "growth medium" compared with secretors. In addition, as a side effect, if the bacterial enzyme which degrades the blood group determinant is membrane bound, it can act as an adhesin in the process of colonization.

7.7 Applications of identification of host receptors and bacterial ligands

Clinical diagnosis and prevention of bacterial infection could be supplemented by knowledge of the specific receptors involved in bacterial binding. For diagnostic purposes, synthetic receptor analogues with an optimized binding property could be linked to a solid matrix for selective uptake and detection of a bacterial ligand from infected samples (urine, blood, etc.). The ideal approach to the control of meningococcal infections would be to block their initial attachment to the mucosal surfaces thereby preventing colonization. Interference with bacterial adherence can be achieved by actively inducing an inhibitory substance (i.e. vaccination) or by passive administration of an inhibitory substance (i.e. receptor analogue).

Non-immunogenic methods such as the use of potent receptor analogues to inhibit ligand attachment to mucosal membrane have been suggested in the case of oral cavity and gastro-intestinal tract infections (Freter, 1980). Since most natural foodstuffs contain sugars, polysaccharides, glycoproteins and glycolipids, many of them have been suggested as a method for reducing bacterial binding to mucosal surfaces when lectin-like interactions are involved. Since the site of meningococcal colonization is the nasopharynx and oropharynx, this approach is of limited use unless the substances could be applied as aerosols or droplets.

Vaccines have been used as means of preventing the spread of meningococcal infection. The meningococcal polysaccharide vaccines have several limitations discussed in section 1.3. These vaccines were found to be highly effective at preventing meningococcal disease, but they are much less effective at eliminating

carriage and at preventing nasopharyngeal colonization (reviewed by Greenwood, 1984). Thus, the methods of vaccination available cannot be relied on to stop the spread of epidemic strains.

Prevention of initiation of infection by microorganisms that use the mucosal surface as their primary location of attachment (such as *N. meningitidis*) is associated with secretory IgA (sIgA). sIgA was demonstrated to be effective in cross-linking particles, preventing adhesion and inactivating toxins (reviewed by Hone and Hackett, 1989). Stimulation of sIgA requires the administration of antigen to mucosal surfaces since an effective sIgA response is not provoked following parenteral vaccination. Therefore, the approach available for meningococcal vaccination, i.e. polysaccharide vaccine given parenteraly, does not stimulate sIgA which is the most important type of antibody in prevention of colonization. In addition, since polysaccharides are T-independent antigens, they are ineffective at inducing immunogenic memory. Thus, booster immunization does not result in an accelerated and enhanced antibody response. For these reasons, many groups have turned their attention towards the development of other types of meningococcal vaccines and other methods for administration.

One method that has been studied is oral vaccination which has been shown to be effective in reducing infection and colonization by *Vibrio cholerae* (reviewed by Hone and Hackett, 1989). Oral administration of a vaccine stimulates primarily the mucosal immune system of the gastro-intestinal tract. It has been established that induction of local antibody production at a given mucosal site does not require the application of the immunizing antigen to the same site (see Underdown and Schiff, 1986). Hence, oral administration of meningococcal vaccine will induce local immunity in the oropharyngeal mucosa.

Bacterial surface antigens as oral vaccines has been examined for enterotox-

igenic strains of *E. coli* (del la Cabada *et al.*, 1981). Rabbits orally immunized with purified CFA/I (colonization factor I) fimbriae of *E. coli* were protected against challenge with virulent strains expressing homologous fimbriae. This protection correlated with increased levels of anti-CFA/I sIgA. In the search for new antigens for a meningococcal vaccine, an outer-membrane protein vaccine has been used in a clinical trial in South Africa (reviewed by Greenwood, 1984). This vaccine was effective in reducing carriage with organisms of homologous serotypes, but the number of clinical cases occurring in the test and control groups was too low to allow conclusions to be drawn about its efficacy in preventing clinical disease.

An alternative method for stimulating a local mucosal immunity has been used for the prevention of viral infections of the respiratory tract mucosa. Internasal administration of a live attenuated influenza virus has been used extensively in the United States and the Soviet Union (Weir, 1988). This method which was found to effectively produce neutralizing sIgA antibodies might be useful as a means of delivering meningococcal vaccines.

The epidemiological studies and the results of this current investigation have indicated possible candidates for a meningococcal vaccine and the population at most risk. It appears that non-secretors are more susceptible to meningococcal infections because they are more likely to carry the bacteria. This differential susceptibility does not appear to be associated with a specific serotype or serogroup of meningococci (see section 1.2.4). In addition, the results of the experiments presented in section 5.3.3, suggest that adsorption of molecules containing Le^a is not limited to a particular isolate of these bacteria. Further studies aimed at identifying the particular receptor on non-secretors might provide a tool for isolating the ligand on the bacteria. Such a ligand would be

a suitable candidate as a vaccine, since it is probably common to a variety of meningococcal strains and since receptor-binding properties of bacteria are likely to be highly conserved. In addition, since this ligand is probably a protein, it will provoke a T-dependent immune response which will ensure the production of immunogenic memory.

This study presents preliminary evidence for a role for blood group determinants as receptors for meningococcal attachment. The results highlight the complexity of studying host factors which play a role in the differential susceptibility of non-secretors. In order to draw definite conclusions as to the role of a given structure in bacterial attachment, further studies should make more use of commercially available purified or synthetic antigens. This approach was applied in experiments described in chapter 6. Extending these investigations could lead to the identification of the cell surface receptor(s) and bacterial ligand(s), which might lead to a new type of meningococcal vaccine.

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Abbreviations

BEC Buccal epithelial cells

BSA Bovine serum albumin

DPBS Dulbecco's phosphate-buffered saline

ELISA Enzyme linked immunosorbent assay

FITC Fluorescein isothiocyanate

HSA Human serum albumin

Le Lewis

Le^a Lewis^a

Le^b Lewis^b

MNYC Modified New York City

NPC Nasopharyngeal cells

OMP Outer membrane protein

OPC Oropharyngeal cells

PBS Phosphate-buffered saline

SDS-PAGE Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Se Secretor

UEAI *Ulex europaeus* lectin I